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Docket No. 70-000410US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

US Patent Application For

RNAi-BASED SENSORS, CAGED INTERFERING RNAS, AND METHODS OF USE THEREOF

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RNAI-BASED SENSORS, CAGED INTERFERING RNAS, AND METHODS OF USE THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is a non-provisional utility patent application claiming priority to and benefit of the following prior provisional patent applications: USSN 60/427,664, filed Nov. 18, 2002, entitled "Photo Activated Sensors, Regulators and Compounds" by Nguyen and McMaster, USSN 60/436,855, filed Dec. 26, 2002, entitled "Caged Sensors, Regulators and Compounds and Uses Thereof" by Nguyen and McMaster,, USSN 60/439,917, filed Jan. 13, 2003, entitled "Caged Sensors, Regulators and Compounds and Uses Thereof" by Nguyen and McMaster, USSN 60/451,177, filed Feb. 27, 2003, entitled "Caged Sensors, Regulators and Compounds and Uses Thereof" by Nguyen et al., USSN 60/456,870, filed Mar. 21, 2003, entitled "Caged Sensors, Regulators and Compounds and Uses Thereof" by Nguyen et al.; USSN 60/484,785, filed July 3, 2003, entitled "RNAi-based Sensors and Methods of Use Thereof" by Nguyen and McMaster, and USSN 60/501,599 filed September 9, 2003, entitled "Caged Sensors, Regulators and Compounds and Uses Thereof" by Nguyen et al., each of which is incorporated herein by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] This invention is in the field of RNA interference (RNAi). The invention provides RNAi-based sensors and methods for detecting and/or quantitating target mRNAs. The invention also relates to caged interfering RNAs (e.g., photoactivatable caged RNAs) and use of such caged interfering RNAs to precisely control, spatially and/or temporally, initiation of RNA interference. The invention also relates to introduction of single- or double-stranded RNAs into cells. Methods and compositions for selectively attenuating expression of a target mRNA by controlling expression of an interfering RNA are also provided.

BACKGROUND OF THE INVENTION

[0003] A number of experimental designs in basic research, clinical diagnosis, drug discovery, and the like involve the detection, and frequently also the quantitation, of a particular mRNA. However, current methods for detecting and/or measuring mRNA transcripts from cells (such as Northern blot, quantitative rt-PCR, microarray, branched

DNA, and in situ hybridization assays) generally require the cells to be lysed or fixed. In addition, most current methods require that mRNA purification and/or reverse transcription be performed. Furthermore, current methods typically involve multi-step processing that contributes to high intra- and inter-assay variation. Consequently, current methods do not provide live, dynamic, and location-specific imaging and measurements of mRNA, because the cells are exposed to environmental changes.

[0004] Cellular assays are critical tools in the drug discovery process and in basic research. In the future, these assays will play a major role in systems biology, permitting the examination of cell structure and function and the determination of a drug compound's ability to enter a cell, the compound's toxicity and its overall efficacy. Advances in imaging technologies, fluorescent probes, and assay automation are predicted to drive the worldwide cellular assays market from an estimated $300 million in 2002 to $500 million in 2007. The most common application for cellular assay technology in drug discovery is target validation and lead identification and optimization. However, the complexity and richness in cellular assay data sets, compared to genomics and proteomics studies, will provide scientists with unparalleled tools to aid discovery efforts throughout the discovery process and for basic research applications.

[0005] To achieve the goal of measuring the spectrum of molecular events in cells, there is definite need for "in cell sensor probes" that quantitatively measure protein (or other) activities, mRNA levels, or the like, directly in cells in a regulated fashion to give real time functional data, without using expression vectors. These "in cell sensor probes" could be used to define pathways in a Parallel Quantitative Biology (PQB) format for systems biology, providing novel regulated cell-based functional screening in a high throughput mode. This invention provides such probes, termed PAC probes (PhotoActivated Cell probes), herein. Probes that comprise interfering RNAs are provided by the current invention. The invention also provides other benefits which will become apparent upon review of the disclosure. A complete understanding of the invention will be obtained upon review of the following.

SUMMARY OF THE INVENTION

[0006] The present invention provides methods, compositions, and kits related to RNAi. For example, the invention provides labeled interfering RNAs that can be used as in

cell sensors to detect and/or quantitate RNAs. Methods using labeled interfering RNAs to detect and/or quantitate RNAs in cells are also provided, as are related kits. The invention also provides caged interfering RNAs (e.g., photoactivatable caged interfering RNAs) and methods of using such caged RNAs to selectively attenuate expression of target genes. In addition, the invention provides methods and compositions for introducing interfering RNAs into cells. Methods and compositions for selectively attenuating expression of a target mRNA by controlling expression of an interfering RNA are also provided.

[0007] A first general class of embodiments provides methods of detecting a target mRNA in a cell. In the methods, a labeled RNA is provided. The labeled RNA comprises an RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of the target mRNA, and at least one label. The labeled RNA is introduced into the cell, whereby the labeled RNA initiates RNA interference of the target mRNA. This results in an initiation-dependent change in a signal output of the label. The signal output, which provides an indication of the presence of the target mRNA in the cell, is detected. In a preferred class of embodiments, the label is a fluorescent label, and the initiation-dependent change in the signal output of the label is a change in fluorescent emission. The methods can optionally be used to quantitate the amount of target mRNA present in the cell.

[0008] In one class of embodiments, the labeled RNA also includes at least one quencher. The label and the quencher are positioned in the RNA such that fluorescent emission by the label is quenched by the quencher. Initiation of RNA interference by the labeled RNA results in unquenching of the label (and thus an increase in the fluorescent emission by the label). In this class of embodiments, the initiation-dependent change in the signal output is thus an increase in the fluorescent emission by the label.

[0009] In a related class of embodiments, the labeled RNA comprises two fluorescent labels, one being a donor and the other being an acceptor. The donor and acceptor are positioned within the RNA such that energy transfer (e.g., FRET) occurs between them (e.g., excitation of the donor results in fluorescence by the acceptor). Initiation of RNA interference by the labeled RNA results in loss of energy transfer between the donor and the acceptor. This can be observed as an increase in fluorescence by the donor or as a decrease in fluorescence by the acceptor. Thus, in a preferred class of

embodiments, the initiation-dependent change in the signal output is a decrease in fluorescent emission by the acceptor following excitation of the donor.

[0010] The RNA can have any of a variety of structures, lengths, and/or the like. Thus, in one class of embodiments, the RNA comprises a first polyribonucleotide comprising the sense strand and a second polyribonucleotide comprising the antisense strand. The RNA can be, e.g., a long double-stranded RNA that is cleaved by Dicer in the cell, or it can be, e.g., an siRNA. For example, the first polyribonucleotide can comprise between 19 and 25 nucleotides, the second polyribonucleotide can comprise between 19 and 25 nucleotides, and the double-stranded region can comprise between 19 and 25 base pairs. The first and second polyribonucleotides can form a duplex over their entire length, or they can have overhangs (e.g., 5' or 3' overhangs; e.g., 21 nt first and second polyribonucleotides can form a 19 bp double-stranded region with 2 nucleotide overhangs, 23 nt polyribonucleotides can form a 21 bp double-stranded region with 2 nucleotide overhangs, and so on). For example, in some embodiments, the first polyribonucleotide and the second polyribonucleotide each comprise a two nucleotide TT 3' overhang (where T is 2'-deoxythymidine). Instead of comprising two polyribonucleotides, in some embodiments, the RNA comprises a self-complementary polyribonucleotide (e.g., a shRNA). The RNA is optionally nuclease resistant.

[0011] The RNA sensors are optionally caged. Caging a sensor, e.g., with a photolabile group, allows the initiation of RNAi, and thus the detection of the target mRNA, to be precisely controlled, temporally and/or spatially. Thus, in one class of embodiments, the labeled RNA further comprises one or more first caging groups associated with the RNA. The first caging groups inhibit (e.g., prevent) the RNA from initiating RNA interference of the target mRNA in the cell. RNA interference of the target mRNA is initiated by exposing the cell to uncaging energy of a first type, whereby exposure to the uncaging energy frees the RNA from inhibition by the first caging groups. The one or more first caging groups associated with the RNA can be covalently or non-covalently attached to the RNA. In a preferred aspect, the one or more first caging groups are photoactivatable (e.g., photolabile).

[0012] Caging permits temporal control over activation of the sensor. For example, the method can include contacting the cell and a test compound and exposing the cell to the uncaging energy at a preselected time point with respect to a time at which the cell and the

test compound are contacted (e.g., to determine if the test compound directly or indirectly affects expression of the target mRNA). Caging also permits spatial control over activation of the sensor. For example, the uncaging energy can be directed at a preselected subset of a cell population comprising the cell.

[0013] In one class of embodiments, the labeled RNA also includes a cellular delivery module, associated with the RNA, that can mediate introduction of the labeled RNA into the cell. In this class of embodiments, introducing the labeled RNA into the cell comprises contacting the cell with the labeled RNA associated with the cellular delivery module.

[0014] The methods can optionally be used to monitor gene expression, for example, induction of transcription of the target mRNA in response to a stimulus. Thus, in one class of embodiments, the methods include stimulating the cell, e.g., by adding a test compound (e.g., a drug, a candidate drug, a receptor agonist or putative agonist, or the like), by changing growth conditions, by adding other cells, etc.

[0015] The methods can be used to examine expression of the target mRNA, e.g., in two different cell populations, one stimulated and one not. Similarly, expression of the target mRNA can be monitored in a single cell (or a single cell population) before and after stimulation of the cell. Thus, in one embodiment, the signal output is detected at a plurality of time points with respect to a time at which the cell is stimulated.

[0016] Another aspect of the invention includes kits related to the methods. For example, one class of embodiments provides a kit for detecting a target mRNA in a cell. The kit includes a labeled RNA and, typically, instructions for using the labeled RNA to detect the presence of the target mRNA in the cell, e.g., packaged in one or more containers. The labeled RNA comprises an RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of the target mRNA. The labeled RNA also comprises at least one label, wherein initiation of RNA interference of the target mRNA by the labeled RNA in the cell results in an initiation-dependent change in a signal output of the label. In addition, it is worth noting that the kit optionally also includes at least one buffer and/or at least one delivery reagent. The kit also optionally includes packaging or instructional materials for such additional reagents.

[0017] An additional class of embodiments also provides a kit for detecting a target mRNA in a cell. In this class of embodiments, the kit comprises a target RNA sensor and a reference RNA sensor, packaged in one or more containers. The target RNA sensor comprises a first RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA, and at least one first label, wherein initiation of RNA interference of the target mRNA by the first RNA in the cell results in an initiation-dependent change in a signal output of the first label. The reference RNA sensor comprises a second RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a reference mRNA, and at least one second label, wherein initiation of RNA interference of the reference mRNA by the second RNA in the cell results in an initiation-dependent change in a signal output of the second label; packaged in one or more containers. Typically, the signal output of the first label is detectably different from the signal output of the second label.

[0018] Yet another aspect of the invention provides compositions related to the methods (e.g., compositions produced by the methods or facilitating use of the methods). For example, one class of embodiments provides a population of labeled RNAs for detecting a target mRNA in a cell. Each labeled RNA comprises an RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of the target mRNA, and at least one label. The label is located a preselected position in the RNA, and initiation of RNA interference of the target mRNA by the labeled RNA in the cell results in an initiation-dependent change in a signal output of the label.

[0019] All of the various optional configurations and features noted for the embodiments above apply here as well, to the extent they are relevant, e.g., for label configurations (e.g., use of fluorescent labels, fluorescent label/quencher, and donor/acceptor combinations), signal output types, RNA configurations (e.g., one or two polyribonucleotides, of various lengths, with or without overhangs, etc.), use of caging groups (e.g., photolabile caging groups), appropriate uncaging energies (light, heat, sonic,

etc.), use of cellular delivery modules (e.g., amphipathic peptides, protein transduction domains, and lipids), and the like. It is worth noting that the composition optionally also includes the target mRNA and/or a cell, e.g., a cell comprising the target mRNA and/or the population.

[0020] In another class of embodiments, the invention provides a composition comprising a target RNA sensor and a reference RNA sensor. The target RNA sensor comprises a first RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA, and at least one first label, wherein initiation of RNA interference of the target mRNA by the first RNA in the cell results in an initiation-dependent change in a signal output of the first label. The reference RNA sensor comprises a second RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a reference mRNA, and at least one second label, wherein initiation of RNA interference of the reference mRNA by the second RNA in the cell results in an initiation-dependent change in a signal output of the second label. Typically, the signal output of the first label is detectably different from the signal output of the second label. All of the various optional configurations and features noted for the embodiments above apply here as well, to the extent they are relevant.

[0021] In one aspect of this invention, caging groups (e.g., photo-labile caging groups) are used to precisely control the timing and/or location of RNA interference. Thus, one general class of embodiments provides a composition comprising a caged interfering RNA. The caged RNA includes an RNA having at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. The caged RNA also includes one or more first caging groups associated with the RNA. The first caging groups inhibit (e.g., prevent) the RNA from initiating RNA interference of the target mRNA in a cell comprising the caged RNA. Removal of or an induced conformational change in the first caging groups permits the RNA to initiate RNA interference of the target mRNA in the cell.

[0022] The RNA can have any of a variety of structures, lengths, and/or the like. Thus, in one class of embodiments, the RNA comprises a first polyribonucleotide comprising the sense strand and a second polyribonucleotide comprising the antisense strand. In other embodiments, the RNA comprises a self-complementary polyribonucleotide (e.g., a hairpin, a shRNA).

[0023] The one or more first caging groups associated with the RNA can be covalently or non-covalently attached to the RNA. In a preferred aspect, the one or more first caging groups are photoactivatable (e.g., photolabile). Other caging groups are removable via input of different uncaging energies; e.g., the one or more caging groups can be removable by sonication or application of heat, or can be removed by a chemical or enzyme.

[0024] The RNA optionally also includes at least one label, wherein initiation of RNA interference of the target mRNA by the labeled RNA in the cell results in an initiation-dependent change in a signal output of the label. In a preferred class of embodiments, the label is a fluorescent label, and the initiation-dependent change in the signal output of the label is a change in fluorescent emission.

[0025] In one class of embodiments, the labeled RNA also includes at least one quencher. The label and the quencher are positioned in the RNA such that fluorescent emission by the label is quenched by the quencher. Initiation of RNA interference by the labeled RNA results in unquenching of the label (and thus an increase in the fluorescent emission by the label). In this class of embodiments, the initiation-dependent change in the signal output is, thus, an increase in the fluorescent emission by the label.

[0026] In a related class of embodiments, the labeled RNA comprises two fluorescent labels, one of which is a donor and the other of which is an acceptor. The donor and acceptor are positioned within the RNA such that energy transfer (e.g., fluorescence resonance energy transfer, or "FRET") occurs between them (e.g., excitation of the donor results in fluorescence by the acceptor). Initiation of RNA interference by the labeled RNA results in loss of energy transfer between the donor and the acceptor. This can be observed as an increase in fluorescence by the donor or as a decrease in fluorescence by the acceptor. Thus, in a preferred class of embodiments, the initiation-dependent change in the signal

output is a decrease in fluorescent emission by the acceptor following excitation of the donor.

[0027] The composition optionally includes the target mRNA. The caged RNA is optionally located inside a cell, e.g., where use of the RNA in a cell is desirable; that is, the composition optionally comprises a cell comprising the caged RNA and/or the target mRNA. In one class of embodiments, the caged RNA also includes a cellular delivery module, associated with the RNA, that can mediate introduction of the caged RNA into the cell.

[0028] Optionally, in the embodiments herein, the caged RNA is bound to a matrix (e.g., electrostatically, covalently, directly or via a linker). In one aspect, the matrix is a surface and the RNA is bound to the surface at a predetermined location within an array comprising other RNAs. In other embodiments, the matrix comprises a bead (e.g., color-coded or otherwise addressable).

[0029] Kits for making the caged RNA (e.g., comprising an RNA, one or more first caging groups, and instructions for assembling the RNA and the first caging groups to form the caged RNA, packaged in one or more containers, and/or one or more first caging groups and instructions for assembling the first caging groups and an RNA supplied by a user of the kit to form the caged RNA, packaged in one or more containers) are also a feature of the invention. Similarly, the invention provides kits for making caged and labeled RNA, e.g., a kit comprising one or more first caging groups, at least one label, and instructions for assembling the first caging groups, at least one label, and an RNA supplied by a user of the kit to form the caged RNA, packaged in one or more containers.

[0030] A related general class of embodiments provides a composition comprising a caged interfering RNA. The caged RNA comprises a single polyribonucleotide strand that comprises an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. The caged RNA also includes one or more first caging groups associated with the RNA. The first caging groups inhibit (e.g., prevent) the RNA from initiating RNA interference of the target mRNA in a cell comprising the caged RNA.

[0031] All of the various optional configurations and features noted for the embodiments above apply here as well, to the extent they are relevant, e.g., for label

configurations (e.g., use of fluorescent labels, fluorescent label/quencher, and donor/acceptor combinations), signal output types, use of caging groups (e.g., photolabile caging groups), appropriate uncaging energies (light, heat, sonic, etc.), use of cellular delivery modules (e.g., amphipathic peptides, protein transduction domains, and lipids), and the like.

[0032] In one class of methods of the invention, methods of selectively attenuating expression of a target gene in a cell are provided. In these methods, a caged RNA is introduced into the cell. The caged RNA can include an RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA corresponding to the target gene. Alternatively, the caged RNA can comprise a single polyribonucleotide strand that comprises an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of the target mRNA. The caged RNA comprises one or more caging groups associated with the RNA, and the caging groups inhibit (e.g., prevent) the RNA from initiating RNA interference of the target mRNA in the cell. RNA interference is initiated by exposing the cell to uncaging energy (e.g., light of a predetermined wavelength), freeing the RNA from inhibition by the caging groups. All of the above optional method variations apply to this method as well. Further, the various composition components noted (particularly the caged RNA embodiments) above can be adapted for use in this method, as appropriate.

[0033] Protein transduction domains can be used to introduce interfering RNAs into cells. Thus, one class of embodiments provides a composition comprising a protein transduction domain covalently attached to an RNA. The RNA can comprise at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. Alternatively, the RNA can comprise a single polyribonucleotide strand comprising an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. The composition is optionally also includes the target mRNA and/or a cell, e.g., a cell comprising the target mRNA and/or the RNA.

[0034] Kits for making the protein transduction domain-linked RNAs are also a feature of the invention. For example, one embodiment provides a kit comprising an RNA, a protein transduction domain, and instructions for assembling the RNA and the protein transduction domain to form the composition, packaged in one or more containers. A related embodiment provides a kit comprising a protein transduction domain and instructions for assembling the protein transduction domain and an RNA supplied by a user of the kit to form the composition, packaged in one or more containers.

[0035] The invention also provides related methods of introducing an RNA into a cell. In the methods, a composition comprising an RNA and a protein transduction domain covalently attached to the RNA is provided. The RNA can comprise at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. Alternatively, the RNA can comprise a single polyribonucleotide strand comprising an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. The composition and the cell are contacted, whereby the protein transduction domain mediates introduction of the RNA into the cell.

[0036] Interfering RNAs can also be introduced into cells by covalently or non-covalently associated lipids. Thus, one class of embodiments provides a composition comprising an RNA and a lipid covalently attached to the RNA. The RNA can comprise at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. Alternatively, the RNA can comprise a single polyribonucleotide strand comprising an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. The lipid can be, e.g., a fatty acid. In one example class of embodiments, the lipid comprises (or, e.g., consists of) a myristoyl group.

[0037] The invention also provides related methods of introducing an RNA into a cell. In the methods, a composition comprising an RNA and a lipid covalently attached to the RNA is provided. The RNA can comprise at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target

mRNA. Alternatively, the RNA can comprise a single polyribonucleotide strand comprising an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. The composition and the cell are contacted, whereby the lipid mediates introduction of the RNA into the cell.

[0038] In one aspect, the invention includes methods of selectively attenuating expression of a target mRNA in a cell. In the methods, one or more vectors that comprise or encode an RNA are introduced into the cell. The RNA comprises at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of the target mRNA. A caged first activation component is also introduced into the cell. The caged first activation component includes one or more caging groups associated with a first activation component. The first activation component directly or indirectly increases expression of the RNA from the one or more vectors, and the one or more caging groups inhibit (e.g., prevent) the first activation component from increasing expression of the RNA. The cell is exposed to uncaging energy (e.g., light of a first wavelength), whereby exposure to the uncaging energy frees the first activation component from inhibition by the caging groups. This results in increased expression of the RNA, which can then initiate RNA interference of the target mRNA.

[0039] Compositions related to the methods are also provided. Thus, one general class of embodiments provides a composition comprising one or more vectors and a caged first activation component. The one or more vectors comprise or encode an RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. The caged first activation component comprises one or more caging groups associated with a first activation component, which first activation component directly or indirectly increases expression of the RNA from the one or more vectors in a cell comprising the one or more vectors and the first activation component, and which one or more caging groups inhibit the first activation component from increasing expression of the RNA in the cell. The composition optionally includes the target mRNA and/or a cell, e.g., a cell comprising the one or more vectors and the caged first activation component and/or the target mRNA.

[0040] The invention also includes additional methods of selectively attenuating expression of a gene in a cell. In the methods, a first caged DNA and a second caged DNA are introduced into the cell. The first caged DNA includes a first DNA encoding an RNA sense strand and one or more caging groups. The second caged DNA comprises a second DNA encoding an RNA antisense strand and one or more caging groups. The presence of the caging groups prevents transcription of the first and second DNAs, the first and second DNAs each comprising at least a portion of the target gene, and the sense and antisense strands being at least partially complementary and able to form a duplex over at least a portion of their lengths. RNA interference is initiated by generating double-stranded RNA by exposing the cell to uncaging energy, whereby exposure to the uncaging energy frees the first and second DNAs from the caging groups and permits transcription of the first and second DNAs to occur. All of the above optional method variations apply to this method as well, to the extent they are relevant. Further, the various composition components noted above can be adapted for use in this method, as appropriate; e.g., use of caging groups (e.g., photolabile caging groups), appropriate uncaging energies (light, heat, sonic, etc.), use of cellular delivery modules (e.g., amphipathic peptides, protein transduction domains, and lipids), and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

[0041] Figure 1 Panels A-I schematically illustrate example interfering RNA sensors. The top strand corresponds to the sense strand and the bottom the antisense strand. Boxes A and B represent either a fluorescent label and a quencher (or vice versa) or a donor and acceptor (or vice versa).

[0042] Figure 2 schematically illustrates multiplexed interfering RNA sensors having different fluorescent label (F)/quencher (Q) combinations.

[0043] Figure 3 schematically illustrates multiplexed interfering RNA sensors, one example with a donor/acceptor combination (F1/F2), one example with a donor/acceptor combination suitable for TR-FRET (APC/Eu), and one example with a fluorescent label/quencher (F5/Q) combination.

[0044] Figure 4 schematically illustrates caged siRNAs with a photolabile linker between the two strands. The top strand corresponds to the sense strand and the bottom the antisense strand.

[0045] Figure 5 schematically illustrates example caged siRNAs. The top strand corresponds to the sense strand and the bottom the antisense strand.

[0046] Figure 6 schematically illustrates example caged siRNAs. The top strand corresponds to the sense strand and the bottom the antisense strand.

[0047] Figure 7 schematically illustrates example siRNAs in which a cellular delivery module is attached to the siRNA by a photolabile linker. The top strand corresponds to the sense strand and the bottom the antisense strand.

[0048] Figure 8 schematically illustrates example siRNAs in which a protein carrier is attached to the siRNA by a photolabile linker. The top strand corresponds to the sense strand and the bottom the antisense strand.

[0049] Figure 9 schematically illustrates an example siRNA in which a protein transduction domain- and endosomal release agent- coated bead is attached to the siRNA by a photolabile linker. The top strand corresponds to the sense strand and the bottom the antisense strand.

[0050] Figure 10 schematically illustrates an example siRNA in which a lipid is attached to the siRNA by a photolabile linker. The top strand corresponds to the sense strand and the bottom the antisense strand.

[0051] Figure 11 schematically illustrates caging of an siRNA in a photolabile vesicle (solid oval) associated with a protein transduction domain (PTD). The siRNA is released when the vesicle dissociates (broken oval) after exposure to light.

[0052] Figure 12 schematically illustrates caged siRNAs that can be used, e.g., as probes for mRNA.

[0053] Figure 13 schematically illustrates caged shRNAs that can be used, e.g., as probes for mRNA.

[0054] Figure 14 schematically depicts a caged siRNA linked with a peptide transport moiety.

[0055] Figure 15 schematically illustrates measurement of mRNA with a FRET siRNA.

[0056] Figure 16 schematically illustrates the use of multiple siRNAs per target gene.

[0057] Figure 17 depicts a flowchart illustrating an example workflow for assays using photoactivatable mRNA sensors in a live cell assay format.

[0058] Figure 18 schematically depicts the detection of splice variants with siRNA sensors that span splice junctions.

[0059] Figure 19 presents the sequence of a portion of human GAPDH (SEQ ID NO:1). The positions of the sense strand of three interfering RNAs against GAPDH (RNAi 1-RNAi 3) are also indicated.

[0060] Figure 20 Panel A schematically illustrates an annealed GAPDH interfering RNA sensor; Panel B schematically illustrates a denatured GAPDH interfering RNA sensor; and Panel C shows fluorescent emission spectra for the antisense strand (curve 1), the sense strand (curve 2), and the annealed strands (curve 3) of a GAPDH interfering RNA sensor.

[0061] Figure 21 shows the GAPDH mRNA level as measured by a bDNA assay at the indicated time points after lipofection of labeled RNAi 1 (Panel A), as compared to a negative control (Panel B, no lipofection reagent).

[0062] Figure 22 compares the percentage knockout of GAPDH expression, as measured by the bDNA assay, for labeled RNAi 1-3.

[0063] Figure 23 shows the results of bDNA assays (RLU, luminescence) compared to FITC signals (FLU) for cells lipofected with the RNAi 1 (Panel A), RNAi 2 (Panel B), and RNAi 3 (Panel C) sensors.

[0064] Figure 24 shows the ratio of the bDNA assay measurement of GAPDH mRNA levels at 20 h/4 h and the ratio of the FITC signal from labeled RNAi's 1-3 at 20 h/4 h.

[0065] Figure 25 Panel A presents a graph of the results of bDNA assays (RLU, relative luminescent units, indicating the GAPDH mRNA level) and a graph of the fluorescent signal (RFU, relative fluorescence units) from the labeled RNAi sensor, at the indicated time points after lipofection of the sensor. Panel B presents a graph of the fluorescent signals against the bDNA assay results.

[0066] Figure 26 schematically illustrates use of an environmentally responsive polymer as a noncovalently associated caging group.

[0067] Figure 27 schematically illustrates use of an environmentally responsive polymer as a covalently associated caging group for an siRNA. The top strand of the RNA corresponds to the sense strand and the bottom the antisense strand.

[0068] Figure 28 Panel A schematically illustrates the caged double-stranded siRNA RNAi 1. Panel B depicts the caged antisense strand of caged RNAi 1 (SEQ ID NO:2).

[0069] Figure 29 Panel A presents a graph of GAPDH expression relative to cyclophilin expression in untransfected cells, cells transfected with RNAi 1, cells transfected with in vitro uncaged caged RNAi 1, cells transfected with caged RNAi 1 but not exposed to UV light, and cells transfected with caged RNAi 1 and exposed to UV light, as measured by a bDNA assay. Panel B presents a graph of relative GAPDH expression levels in cells transfected with RNAi 1, transfected with in vitro uncaged caged RNAi 1, transfected with caged RNAi 1 but not exposed to UV light, and transfected with caged RNAi 1 and exposed to UV light, as measured by a bDNA assay and normalized to the expression level in cells transfected with RNAi 1.

[0070] Figure 30 schematically illustrates induction of expression of an interfering RNA by uncaging of a first activation component, tetracycline in this example.

[0071] Figure 31 schematically illustrates induction of expression of an interfering RNA by uncaging of a first activation component, IP3 in this example.

DEFINITIONS

[0072] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. The following definitions supplement those in the art and are directed to the current application and are not to be imputed to any related or unrelated case, e.g., to any commonly owned patent or application. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein. Accordingly, the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0073] As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a protein" includes a plurality of proteins; reference to "a cell" includes mixtures of cells, and the like.

[0074] An "antisense strand" is a nucleic acid strand comprising a sequence complementary to that of a given mRNA, while a "sense strand" is a nucleic acid strand comprising a sequence corresponding to that of the mRNA.

[0075] "Attenuating" expression of a target gene refers to decreasing the level of expression of the gene, e.g., as compared to the level in the absence of a relevant interfering RNA.

[0076] A "caging group" is a moiety that can be employed to reversibly block, inhibit, or interfere with the activity (e.g., the biological activity) of a molecule (e.g., a polypeptide, a nucleic acid, a small molecule, a drug, etc.). The caging groups can, e.g., physically trap an active molecule inside a framework formed by the caging groups. Typically, however, one or more caging groups are associated (covalently or noncovalently) with the molecule but do not necessarily surround the molecule in a physical cage. For example, a single caging group covalently attached to an amino acid side chain required for the catalytic activity of an enzyme can block the activity of the enzyme; the enzyme would thus be caged even though not physically surrounded by the caging group. Caging groups can be, e.g., relatively small moieties such as carboxyl nitrobenzyl, 2-nitrobenzyl, nitroindoline, hydroxyphenacyl, DMNPE, or the like, or they can be, e.g., large bulky moieties such as a protein or a bead. Caging groups can be removed from a molecule, or their interference with the molecule's activity can be otherwise reversed or reduced, by exposure to an appropriate type of uncaging energy and/or exposure to an uncaging chemical, enzyme, or the like.

[0077] A "photoactivatable" or "photoactivated" caging group is a caging group whose blockage, inhibition of, or interference with the activity of a molecule with which the photoactivatable caging group is associated can be reversed or reduced by exposure to light of an appropriate wavelength. For example, exposure to light can disrupt a network of caging groups physically surrounding the molecule, reverse a noncovalent association with the molecule, trigger a conformational change that renders the molecule active even though

still associated with the caging group, or cleave a photolabile covalent attachment to the molecule.

[0078] A "photolabile" caging group is one whose covalent attachment to a molecule is reversed (cleaved) by exposure to light of an appropriate wavelength. The photolabile caging group can be, e.g., a relatively small moiety such as carboxyl nitrobenzyl, 2-nitrobenzyl, nitroindoline, hydroxyphenacyl, DMNPE, or the like, or it can be, e.g., a relatively bulky group (e.g. a macromolecule, a protein) covalently attached to the molecule by a photolabile linker (e.g., a polypeptide linker comprising a 2-nitrophenyl glycine residue).

[0079] A "cellular delivery module" or "cellular delivery agent" is a moiety that can mediate introduction into a cell of a molecule with which the module is associated (covalently or noncovalently).

[0080] The term "eukaryote" refers to organisms belonging to the phylogenetic domain Eucarya such as animals (e.g., mammals, insects, reptiles, birds, etc.), ciliates, plants, fungi (e.g., yeasts, etc.), flagellates, microsporidia, protists, etc. Additionally, the term "prokaryote" refers to non-eukaryotic organisms belonging to the Eubacteria (e.g., *Escherichia coli*, *Thermus thermophilus*, etc.) and Archaea (e.g., *Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum*, *Halobacterium* species, etc.) phylogenetic domains.

[0081] "Expression of a gene" or "expression of a nucleic acid" means transcription of DNA into RNA (optionally including modification of the RNA, e.g., splicing) and/or translation of encoded RNA (e.g., mRNA) into a polypeptide (possibly including subsequent modification of the polypeptide, e.g., post-translational modification), as indicated by the context.

[0082] The term "gene" is used broadly to refer to any nucleic acid associated with a biological function. Genes typically include coding sequences and/or the regulatory sequences required for expression of such coding sequences.

[0083] A "label" is a moiety that facilitates detection of a molecule. Common labels in the context of the present invention include fluorescent, luminescent, and/or colorimetric labels. Suitable labels include radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents

teaching the use of such labels include U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Many labels are commercially available and can be used in the context of the invention.

[0084] The term "nucleic acid" encompasses any physical string of monomer units that can be corresponded to a string of nucleotides, including a polymer of nucleotides (e.g., a typical DNA or RNA polymer), PNAs, modified oligonucleotides (e.g., oligonucleotides comprising nucleotides that are not typical to biological RNA or DNA, such as 2'-O-methylated oligonucleotides), and/or the like. A nucleic acid can be, e.g., single-stranded or double-stranded. Unless otherwise indicated, a particular nucleic acid sequence of this invention optionally comprises or encodes complementary sequences, in addition to any sequence explicitly indicated. A nucleic acid (e.g., a polyribonucleotide, a double-stranded RNA, or the like) of this invention is optionally nuclease resistant.

[0085] A nucleic acid that is "nuclease resistant" or "resistant to nuclease activity" is cleaved more slowly under typical reaction conditions for a given nuclease (e.g., a 5' to 3' nuclease and/or an endonuclease) than is a corresponding nucleic acid comprising only the four conventional deoxyribonucleotides (A, T, G, and/or C), or the four conventional ribonucleotides (U, A, G, and/or C), and phosphodiester linkages. For example, nucleic acids that incorporate 2'O methylated nucleotides are typically more nuclease resistant than nucleic acids that incorporate only conventional nucleotides. Many such modifications that impart nuclease resistance are known and can be adapted to the present invention.

[0086] An "oligonucleotide" or "polynucleotide" is a polymer comprising two or more nucleotides. (For example, a "polyribonucleotide" is a polymer comprising two or more ribonucleotides.) The polymer can additionally comprise non-nucleotide elements such as labels, quenchers, blocking groups, or the like. The nucleotides of the oligonucleotide can be deoxyribonucleotides, ribonucleotides and/or nucleotide analogs, can be natural or non-natural, and can be unsubstituted, unmodified, substituted or modified. The nucleotides can be linked by phosphodiester bonds, or by phosphorothioate linkages, methylphosphonate linkages, boranophosphate linkages, or the like.

[0087] The "5' end" of a polynucleotide refers to the nucleotide located at the 5' terminus of the polynucleotide. A moiety "attached at the 5' end" of the polynucleotide can thus be attached to any part of the 5' terminal nucleotide, e.g., the terminal 5' phosphate or

hydroxyl, the base, or the ribose. Similarly, the "3' end" of a polynucleotide refers to the nucleotide located at the 3' terminus of the polynucleotide. A moiety "attached at the 3' end" of the polynucleotide can thus be attached to any part of the 3' terminal nucleotide, e.g., the terminal 3' hydroxyl, the base, or the ribose.

[0088] A "subcellular delivery module" or "subcellular delivery agent" is a moiety that can mediate delivery and/or localization of an associated molecule to a particular subcellular location (e.g., a subcellular compartment, a membrane, and/or neighboring a particular macromolecule). The subcellular delivery module can be covalently or noncovalently associated with the molecule. Subcellular delivery modules include, e.g., peptide tags such as a nuclear localization signal or mitochondrial matrix-targeting signal.

[0089] A "synthetic oligonucleotide" or a "chemically synthesized oligonucleotide" is an oligonucleotide made through in vitro chemical synthesis, as opposed to an oligonucleotide made either in vitro or in vivo by a template-directed, enzyme-dependent reaction.

[0090] A "polypeptide" is a polymer comprising two or more amino acid residues (e.g., a peptide or a protein). The polymer can additionally comprise non-amino acid elements such as labels, quenchers, blocking groups, or the like and can optionally comprise modifications such as glycosylation or the like. The amino acid residues of the polypeptide can be natural or non-natural and can be unsubstituted, unmodified, substituted or modified.

[0091] A "protein transduction domain" is a polypeptide sequence that can mediate introduction of a covalently associated molecule into a cell. Protein transduction domains are typically short peptides (e.g., often less than about 16 residues). Example protein transduction domains have been derived from the HIV-1 protein Tat, the herpes simplex virus protein VP22, and the *Drosophila* protein antennapedia; model protein transduction domains have also been designed.

[0092] A "quencher" is a moiety that alters a property of a label (typically, a fluorescent label) when it is in proximity to the label. The quencher can actually quench an emission, but it does not have to, i.e., it can simply alter some detectable property of the label, or, when proximal to the label, cause a different detectable property than when not proximal to the label. A quencher can be e.g., an acceptor fluorophore that operates via energy transfer and re-emits the transferred energy as light; other similar quenchers, called

"dark quenchers," do not re-emit transferred energy via fluorescence. A variety of labels and quenchers are found in Haughland (2003) Handbook of Fluorescent Probes and Research Products Ninth Edition, available from Molecular Probes. A straightforward discussion of FRET can be found in the Handbook at page 25-26 and the references cited therein.

[0093] A "target mRNA" is an mRNA that is to be detected and/or whose expression is to be affected. A "target gene" is a gene whose expression is to be detected (e.g., one or more corresponding mRNAs are to be detected) and/or whose expression is to be affected. A target gene can be, e.g., an endogenous gene or a heterologous gene (e.g., a gene introduced into the cell through infection by a pathogen, or a gene introduced through recombinant means). A target gene can be, e.g., a constitutively expressed gene or an inducible gene; similarly, a target mRNA can be, e.g., a constitutively expressed mRNA or an inducible mRNA.

[0094] "Uncaging energy" is energy that removes one or more caging groups from a caged molecule (or otherwise reverses the caging groups' blockage of the molecule's activity). Uncaging energy can be supplied, e.g., by light, sonication, a heat source, a magnetic field, or the like, as appropriate for the particular caging group(s).

[0095] The term "vector" refers to a means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include plasmids, viruses, bacteriophage, pro-viruses, phagemids, transposons, and artificial chromosomes, and the like, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that are not autonomously replicating. Most commonly, the vectors of the present invention are plasmids.

[0096] A variety of additional terms are defined or otherwise characterized herein.

DETAILED DESCRIPTION

[0097] The term "RNA interference" ("RNAi," sometimes called RNA-mediated interference, post-transcriptional gene silencing, or quelling) refers to a phenomenon in which the presence of double-stranded RNA in a cell results in inhibition of expression of a

gene comprising a sequence identical, or nearly identical, to that of the double-stranded RNA. The double-stranded RNA responsible for inducing RNAi is called an "interfering RNA." Expression of the gene is inhibited by the mechanism of RNAi as described below, in which the presence of the interfering RNA results in degradation of mRNA transcribed from the gene and thus in decreased levels of the mRNA and any encoded protein.

[0098] The mechanism of RNAi has been and is being extensively investigated in a number of eukaryotic organisms and cell types. See, for example, the following reviews: McManus and Sharp (2002) "Gene silencing in mammals by small interfering RNAs" Nature Reviews Genetics 3:737-747; Hutvagner and Zamore (2002) "RNAi: Nature abhors a double strand" Curr Opin Genet & Dev 200:225-232; Hannon (2002) "RNA interference" Nature 418:244-251; Agami (2002) "RNAi and related mechanisms and their potential use for therapy" Curr Opin Chem Biol 6:829-834; Tuschl and Borkhardt (2002) "Small interfering RNAs: A revolutionary tool for the analysis of gene function and gene therapy" Molecular Interventions 2:158-167; Nishikura (2001) "A short primer on RNAi: RNA-directed RNA polymerase acts as a key catalyst" Cell 107:415-418; and Zamore (2001) "RNA interference: Listening to the sound of silence" Nature Structural Biology 8:746-750. RNAi is also described in the patent literature; see, e.g., CA 2359180 by Kreutzer and Limmer entitled " Method and medicament for inhibiting the expression of a given gene"; WO 01/68836 by Beach et al. entitled "Methods and compositions for RNA interference"; WO 01/70949 by Graham et al. entitled "Genetic silencing"; and WO 01/75164 by Tuschl et al. entitled "RNA sequence-specific mediators of RNA interference."

[0099] In brief, double-stranded RNA introduced into a cell (e.g., into the cytoplasm) is processed, for example by an RNAse III-like enzyme called Dicer, into shorter double-stranded fragments called small interfering RNAs (siRNAs, also called short interfering RNAs). The length and nature of the siRNAs produced is dependent on the species of the cell, although typically siRNAs are 21-25 nucleotides long (e.g., an siRNA may have a 19 base pair duplex portion with two nucleotide 3' overhangs at each end). Similar siRNAs can be produced in vitro (e.g., by chemical synthesis or in vitro transcription) and introduced into the cell to induce RNAi. The siRNA becomes associated with an RNA-induced silencing complex (RISC). Separation of the sense and antisense strands of the siRNA, and interaction of the siRNA antisense strand with its target mRNA

through complementary base-pairing interactions, optionally occurs. Finally, the mRNA is cleaved and degraded.

[0100] Expression of a target gene in a cell can thus be specifically inhibited by introducing an appropriately chosen double-stranded RNA into the cell. Guidelines for design of suitable interfering RNAs are known to those of skill in the art. For example, interfering RNAs are typically designed against exon sequences, rather than introns or untranslated regions. Characteristics of high efficiency interfering RNAs may vary by cell type. For example, although siRNAs may require 3' overhangs and 5' phosphates for most efficient induction of RNAi in Drosophila cells, in mammalian cells blunt ended siRNAs and/or RNAs lacking 5' phosphates can induce RNAi as effectively as siRNAs with 3' overhangs and/or 5' phosphates (see, e.g., Czauderna et al. (2003) "Structural variations and stabilizing modifications of synthetic siRNAs in mammalian cells" Nucl Acids Res 31:2705-2716). As another example, since double-stranded RNAs greater than 30-80 base pairs long activate the antiviral interferon response in mammalian cells and result in non-specific silencing, interfering RNAs for use in mammalian cells are typically less than 30 base pairs (for example, Elbashir et al. (2001) "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells" Nature 411:494-498 and Elbashir et al. (2002) "Analysis of gene function in somatic mammalian cells using small interfering RNAs" Methods 26:199-213 describe the use of 21 nucleotide siRNAs to specifically inhibit gene expression in mammalian cell lines). The sense and antisense strands of a siRNA are typically, but not necessarily, completely complementary to each other over the double-stranded region of the siRNA (excluding any overhangs). The antisense strand is typically completely complementary to the target mRNA over the same region, although some nucleotide substitutions can be tolerated (e.g., a one or two nucleotide mismatch between the antisense strand and the mRNA can still result in RNAi, although at reduced efficiency). The ends of the double-stranded region are typically more tolerant to substitution than the middle; for example, as little as 15 bp of complementarity between the antisense strand and the target mRNA in the context of a 21 mer with a 19 bp double-stranded region has been shown to result in a functional siRNA (see, e.g., Czauderna et al. (2003) "Structural variations and stabilizing modifications of synthetic siRNAs in mammalian cells" Nucl Acids Res 31:2705-2716). Any overhangs can but need not be

complementary to the target mRNA; for example, TT (two 2'-deoxythymidines) overhangs are frequently used to reduce synthesis costs.

[0101] Although double-stranded RNAs (e.g., double-stranded siRNAs) were initially thought to be required to initiate RNAi, several recent reports indicate that the antisense strand of such siRNAs is sufficient to initiate RNAi. Single-stranded antisense siRNAs can initiate RNAi through the same pathway as double-stranded siRNAs (as evidenced, for example, by the appearance of specific mRNA endonucleolytic cleavage fragments). As for double-stranded interfering RNAs, characteristics of high-efficiency single-stranded siRNAs may vary by cell type (e.g., a 5' phosphate may be required on the antisense strand for efficient induction of RNAi in some cell types, while a free 5' hydroxyl is sufficient in other cell types capable of phosphorylating the hydroxyl). See, e.g., Martinez et al. (2002) "Single-stranded antisense siRNAs guide target RNA cleavage in RNAi" Cell 110:563-574; Amarzguioui et al. (2003) "Tolerance for mutations and chemical modifications in a siRNA" Nucl. Acids Res. 31:589-595; Holen et al. (2003) "Similar behavior of single-strand and double-strand siRNAs suggests that they act through a common RNAi pathway" Nucl. Acids Res. 31:2401-2407; and Schwarz et al. (2002) Mol. Cell 10:537-548.

[0102] Due to currently unexplained differences in efficiency between siRNAs corresponding to different regions of a given target mRNA, several siRNAs are typically designed and tested against the target mRNA to determine which siRNA is most effective. Interfering RNAs can also be produced as small hairpin RNAs (shRNAs, also called short hairpin RNAs), which are processed in the cell into siRNA-like molecules that initiate RNAi.

[0103] The present invention provides a number of novel methods, compositions, and kits related to RNAi. For example, the invention provides methods in which a labeled interfering RNA is used as an in cell sensor to detect and/or quantitate a target mRNA. The labeled RNA includes a label whose signal output changes when the labeled RNA initiates RNA interference (e.g., if the target mRNA is present in the cell). The labeled RNA sensor is optionally caged to permit temporal and/or spatial control over activation of the sensor. Related kits, systems, and compositions (e.g., comprising labeled interfering RNAs for use as in cell sensors) are also provided.

[0104] As another example, the invention also provides caged interfering RNAs (e.g., photoactivatable interfering RNAs). Such a caged RNA includes, e.g., one or more caging groups that inhibit (e.g., prevent) the RNA from initiating RNA interference and whose removal or change in conformation permits the RNA to initiate RNA interference. Kits for making the caged RNA and kits and systems comprising the caged RNA are also features of the invention, as are methods of using a caged interfering RNA to selectively attenuate expression of a target gene in a cell. Using a caged interfering RNA in the methods permits the initiation of RNAi to be precisely controlled, temporally and/or spatially.

[0105] As yet another example, the invention provides novel methods and compositions for introducing interfering RNAs into cells. An interfering RNA can be covalently attached to a protein transduction domain and/or to a lipid (e.g., a myristoyl group) that can mediate its introduction into a cell. Methods of introducing an interfering RNA into a cell, by contacting the cell with the protein transduction domain- and/or lipid-linked interfering RNA, are also provided. The covalent attachment between the protein transduction domain or lipid and the RNA is optionally reversible (e.g., the attachment can be a photolabile linker or a disulfide bond).

[0106] As yet another example, the invention provides additional methods of selectively attenuating expression of a target gene in a cell. In some embodiments, transcription of an interfering RNA is controlled by use of a caged activation component. In other embodiments, caged DNAs encoding an interfering RNA are introduced into a cell and then uncaged to permit transcription of the interfering RNA. The following sections describe the invention in more detail.

INTERFERING RNA SENSORS

[0107] The use of double-stranded RNAs to attenuate expression of target genes by RNAi has been well described, including, e.g., the use of labels on siRNAs to localize the siRNAs in transfected cells (e.g., to the cytoplasm, endosome, nucleus, or the like; see, e.g., Byrom et al. "Visualizing siRNA in mammalian cells: Fluorescence analysis of the RNAi effect" Ambion TechNotes 9(3)). Such labels are typically "nonfunctional labels"; that is, they provide a signal output which remains constant regardless of whether the labeled siRNA initiates RNAi (e.g., interacts with RISC and/or the target mRNA). This invention,

however, provides novel methods of using labeled interfering RNAs as in cell sensors to detect expression of target genes. In the methods, the labels are "functional labels"; that is, the labels provide a signal output that is dependent on whether the labeled interfering RNA (e.g., the labeled siRNA) initiates RNAi of the target mRNA (e.g., interacts with RISC and/or the target mRNA and undergoes strand separation, leading to endonucleolytic cleavage of the target mRNA). Compositions, systems, and kits related to the methods are also provided. The methods, compositions, systems, and kits overcome the above noted difficulties associated with current methods of detecting and/or quantitating mRNA transcripts from cells, by enabling detection and/or quantitation of mRNA in living cells and without requiring mRNA purification, reverse transcription, or cell lysis or fixation.

Methods

[0108] A first general class of embodiments provides methods of detecting a target mRNA in a cell. In the methods, a labeled RNA is provided. The labeled RNA comprises an RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of the target mRNA, and at least one label. The labeled RNA is introduced into the cell, whereby the labeled RNA initiates RNA interference of the target mRNA. This results in an initiation-dependent change in a signal output of the label. The signal output, which provides an indication of the presence of the target mRNA in the cell, is detected. The target mRNA can be, for example, a constitutively expressed mRNA or an mRNA whose expression is inducible.

[0109] It is worth noting that initiation of RNAi of the target mRNA by the labeled RNA can, but need not, result in a substantial attenuation of expression of the target mRNA. For example, expression of the target mRNA can be unaffected, or expression of the target mRNA can be decreased by at least about 0.001%, at least about 0.01%, at least about 0.1%, at least about 1%, at least about 5%, at least about 10%, at least about 25%, at least about 50%, or at least about 75% or more, or can even be reduced to an undetectable level.

[0110] In a preferred class of embodiments, the label is a fluorescent label, and the initiation-dependent change in the signal output of the label is a change in fluorescent emission. The methods can optionally be used to quantitate the amount of target mRNA present in the cell. For example, the intensity of the fluorescent emission can be measured. The intensity provides an indication of the quantity of the target mRNA present in the cell.

[0111] In one class of embodiments, the labeled RNA also includes at least one quencher. The label and the quencher are positioned in the RNA such that fluorescent emission by the label is quenched by the quencher. Initiation of RNA interference by the labeled RNA results in unquenching of the label (and thus an increase in the fluorescent emission by the label). In this class of embodiments, the initiation-dependent change in the signal output is thus an increase in the fluorescent emission by the label. For example, the label and quencher can be positioned on opposite strands, in close enough proximity to each other that the label is quenched until the sense and antisense strands are separated.

[0112] In a related class of embodiments, the labeled RNA comprises two fluorescent labels, one being a donor and the other being an acceptor. The donor and acceptor are positioned within the RNA such that energy transfer (e.g., FRET) occurs between them (e.g., excitation of the donor results in fluorescence by the acceptor). Initiation of RNA interference by the labeled RNA results in loss of energy transfer between the donor and the acceptor. This can be observed as an increase in fluorescence by the donor or as a decrease in fluorescence by the acceptor. Thus, in a preferred class of embodiments, the initiation-dependent change in the signal output is a decrease in fluorescent emission by the acceptor following excitation of the donor. As in the preceding embodiments, the donor and acceptor can, for example, be positioned on opposite strands in close enough proximity to each other that energy transfer occurs until the sense and antisense strands are separated.

[0113] The RNA can have any of a variety of structures, lengths, and/or the like. Thus, in one class of embodiments, the RNA comprises a first polyribonucleotide comprising the sense strand and a second polyribonucleotide comprising the antisense strand. The RNA can be, e.g., a long double-stranded RNA that is cleaved by Dicer in the cell, or it can be, e.g., an siRNA. For example, the first polyribonucleotide can comprise between 19 and 25 nucleotides, the second polyribonucleotide can comprise between 19 and 25 nucleotides, and the double-stranded region can comprise between 19 and 25 base pairs. The first and second polyribonucleotides can form a duplex over their entire length, or they can have overhangs (e.g., 5' or 3' overhangs; e.g., 21 nt first and second polyribonucleotides can form a 19 bp double-stranded region with 2 nucleotide overhangs, 23 nt polyribonucleotides can form a 21 bp double-stranded region with 2 nucleotide overhangs, and so on). For example, in some embodiments, the first polyribonucleotide and the second

polyribonucleotide each comprise a two nucleotide TT 3' overhang (where T is 2'-deoxythymidine). The RNA is optionally nuclease resistant and optionally comprises one or more deoxyribonucleotides one or more PNA monomers, and/or one or more modified nucleotides (e.g., 2'-methyl or 2'-O-allyl ribonucleotides) or internucleotide linkages (e.g., phosphorothioate linkages).

[0114] As described in greater detail below, the RNA sensors can be, and in several embodiments are, caged. Thus, in some embodiments, at least one caging group is associated with the RNA. For example, at least one caging group can be covalently attached to a 5' hydroxyl or a 5' phosphate of the second polyribonucleotide. Since this 5' hydroxyl or phosphate is useful for an siRNA to initiate RNAi (Czauderna et al. (2003) "Structural variations and stabilizing modifications of synthetic siRNAs in mammalian cells" Nucl Acids Res 31:2705-2716), caging the 5' hydroxyl or phosphate of the antisense strand permits the sensor to be uncaged and activated in a controlled manner.

[0115] In one class of embodiments, in which the first and second polyribonucleotides comprise 19-25 nt, the label is a fluorescent label, and the RNA further comprises at least one quencher. The label and the quencher are positioned in the RNA such that fluorescent emission by the label is quenched by the quencher, and initiation of RNA interference by the labeled RNA results in unquenching of the label. The initiation-dependent change in the signal output is thus an increase in the fluorescent emission by the label.

[0116] The label and quencher can be attached to a nucleic acid of the invention at essentially any suitable position(s), e.g., at the 3' end, at the 5' end, and/or within either or both the first and second polyribonucleotides. For example, the label can be attached to the first polyribonucleotide and the quencher attached to the second polyribonucleotide, or the label can be attached to the second polyribonucleotide and the quencher attached to the first polyribonucleotide. For example, one of the label and the quencher can be located within three nucleotides of the 3' end of the first polyribonucleotide and the other of the label and the quencher can be located within three nucleotides of the 3' end of the second polyribonucleotide (see, e.g., Figure 1 Panels A-C). As another example, one of the label and the quencher can be located within three nucleotides of the 3' end of the first polyribonucleotide and the other of the label and the quencher can be located within three nucleotides of the 5' end of the second polyribonucleotide (see, e.g., Figure 1 Panels D and

G). As yet another example, one of the label and the quencher can be located within three nucleotides of the 5' end of the first polyribonucleotide and the other of the label and the quencher can be located within three nucleotides of the 3' end of the second polyribonucleotide (see, e.g., Figure 1 Panels E, F and H). As yet another example, one of the label and the quencher can be located within three nucleotides of the 5' end of the first polyribonucleotide and the other of the label and the quencher can be located within three nucleotides of the 5' end of the second polyribonucleotide (see, e.g., Figure 1 Panel I). In other examples, the label and/or quencher can be in the middle of the RNA; e.g., one of the label and the quencher can be located more than three nucleotides from the 5' end and more than three nucleotides from the 3' end of the first polyribonucleotide, and the other of the label and the quencher can be located more than three nucleotides from the 5' end and more than three nucleotides from the 3' end of the second polyribonucleotide, or, one of the label and the quencher can be located within three nucleotides of the 5' end or the 3' end of the first or second polyribonucleotide and the other of the label and the quencher can be located more than three nucleotides from the 5' end and more than three nucleotides from the 3' end of the opposite polyribonucleotide. In one example embodiment, the label is attached at the 3' end of the first polyribonucleotide and the quencher is attached at the 3' end of the second polyribonucleotide. In a related example embodiment, the quencher is attached at the 3' end of the first polyribonucleotide and the label is attached at the 3' end of the second polyribonucleotide. In yet another example, one of the label and the quencher is attached at the 5' end of the first polyribonucleotide and the other of the label and the quencher is attached at the 3' end of the second polyribonucleotide.

[0117] Techniques for determining and verifying suitable positions for the label and quencher are well known in the art. For example, the label and quencher are typically positioned such that they do not substantially reduce RNAi of the target mRNA as compared to an otherwise identical RNA lacking the label and quencher (e.g., the label and quencher preferably do not interfere with siRNA binding to RISC, strand separation of the siRNA, or binding of the antisense strand to the target mRNA). For example, if the quencher and label are located in the middle of the double-stranded region, the quencher can be attached to a nucleotide one or more nucleotides removed from the complement of the nucleotide to which the fluorescent label is attached. As another example, although overhangs may not be necessary for siRNA function, 3' and/or 5' overhangs of one, two,

three, four, or more nucleotides can optionally be used to position a quencher or label such that it does not interfere with RISC binding to the sensor (see, e.g., Figure 1 panels G and H).

[0118] In a related class of embodiments in which the first and second polyribonucleotides comprise 19-25 nt, the labeled RNA comprises two fluorescent labels, one of which is a donor and the other of which is an acceptor. The donor and acceptor are positioned within the RNA such that energy transfer occurs between them. Initiation of RNA interference by the labeled RNA results in loss of energy transfer between the donor and the acceptor. The initiation-dependent change in signal output can thus be, e.g., a decrease in fluorescent emission by the acceptor following excitation of the donor.

[0119] The donor and acceptor can be attached at essentially any suitable positions, e.g., at the 3' end, at the 5' end, and/or within either or both the first and second polyribonucleotides. For example, the donor can be attached to the first polyribonucleotide and the acceptor to the second polyribonucleotide, or the donor can be attached to the second polyribonucleotide and the acceptor to the first polyribonucleotide. For example, one of the donor and the acceptor can be located within three nucleotides of the 3' end of the first polyribonucleotide and the other of the donor and the acceptor can be located within three nucleotides of the 3' end of the second polyribonucleotide (see, e.g., Figure 1 Panels A-C). As another example, one of the donor and the acceptor can be located within three nucleotides of the 3' end of the first polyribonucleotide and the other of the donor and the acceptor can be located within three nucleotides of the 5' end of the second polyribonucleotide (see, e.g., Figure 1 Panels D and G). As yet another example, one of the donor and the acceptor can be located within three nucleotides of the 5' end of the first polyribonucleotide and the other of the donor and the acceptor can be located within three nucleotides of the 3' end of the second polyribonucleotide (see, e.g., Figure 1 Panel E, F and H). As yet another example, one of the donor and the acceptor can be located within three nucleotides of the 5' end of the first polyribonucleotide and the other of the donor and the acceptor can be located within three nucleotides of the 5' end of the second polyribonucleotide (see, e.g., Figure 1 Panel I). In other examples, the donor and/or acceptor can be in the middle of the RNA; e.g., one of the donor and the acceptor can be located more than three nucleotides from the 5' end and more than three nucleotides from the 3' end of the first polyribonucleotide, and the other of the donor and the acceptor can be

located more than three nucleotides from the 5' end and more than three nucleotides from the 3' end of the second polyribonucleotide, or, one of the donor and the acceptor can be located within three nucleotides of the 5' end or the 3' end of the first or second polyribonucleotide and the other of the donor and the acceptor can be located more than three nucleotides from the 5' end and more than three nucleotides from the 3' end of the opposite polyribonucleotide. In one example embodiment, the donor is attached at the 3' end of the first polyribonucleotide and the acceptor is attached at the 3' end of the second polyribonucleotide. In a related example embodiment, the acceptor is attached at the 3' end of the first polyribonucleotide and the donor is attached at the 3' end of the second polyribonucleotide. In yet another example, one of the donor and the acceptor is attached at the 5' end of the first polyribonucleotide and the other of the donor and the acceptor is attached at the 3' end of the second polyribonucleotide. Techniques for determining and verifying suitable positions for the donor and acceptor are well known in the art.

[0120] Instead of comprising two polyribonucleotides, in some embodiments, the RNA of interest comprises a self-complementary polyribonucleotide (e.g., a shRNA). Label/quencher or acceptor/donor combinations can be similarly positioned within the self-complementary polyribonucleotide.

[0121] As noted, the length of the RNA can vary. For example, the double-stranded region can comprise fewer than about 1500 base pairs, fewer than about 1000 base pairs, fewer than about 500 base pairs, fewer than about 250 base pairs, fewer than about 150 base pairs, fewer than about 80 base pairs, fewer than about 50 base pairs, fewer than about 30 base pairs, or fewer than about 25 base pairs.

[0122] As noted, the RNA sensors can optionally be caged. Caging a sensor, e.g., with a photolabile group, allows the initiation of RNAi, and thus the detection of the target mRNA, to be precisely controlled, temporally and/or spatially. This provides a number of advantages. For example, a caged RNA sensor can be introduced into a cell, e.g., by lipofection. The cell can be permitted to recover from the manipulations necessary to introduce the sensor before uncaging of the sensor permits initiation of RNAi and detection of the target transcript. As another example, until the sensor is uncaged, the interfering RNA exerts no effect on the cell. As yet another example, caging the interfering RNA can protect it from nucleases and thus extend its half-life.

[0123] Thus, in one class of embodiments, the labeled RNA further comprises one or more first caging groups associated with the RNA. The first caging groups inhibit the RNA from initiating RNA interference of the target mRNA in the cell. RNA interference of the target mRNA is initiated by exposing the cell to uncaging energy of a first type, whereby exposure to the uncaging energy frees the RNA from inhibition by the first caging groups.

[0124] The first caging groups can inhibit the RNA from initiating RNA interference of the target mRNA by at least about 25%, at least about 30%, at least about 35%, at least about 50%, at least about 75%, at least about 90%, at least about 95%, or at least about 98%, as compared to the RNA in the absence of the first caging groups. For example, if introduction of an siRNA into a cell decreases expression of its target mRNA to 10% of normal (i.e., expression in a cell not comprising the siRNA), then introduction of the corresponding caged siRNA into a cell would decrease expression to 55% of normal if the caging groups inhibit the RNA from initiating RNA interference by 50% (under equivalent conditions). In one class of embodiments, the first caging groups prevent the RNA from initiating RNA interference of the target mRNA (i.e., introduction of the caged RNA into a cell has no effect on expression of the target mRNA). Removal of or an induced conformational change in the first caging groups typically permits the RNA to initiate RNA interference of the target mRNA.

[0125] The one or more first caging groups associated with the RNA can be covalently attached to or non-covalently associated with the RNA. See, e.g., Figures 5 and 6 for a few of the possible examples of sites of attachment of the caging groups (e.g., at one or more bases, riboses, phosphate groups and/or terminal hydroxyls, within and/or at the end of either or both strands of the RNA). In one embodiment, the RNA comprises a first polyribonucleotide comprising the sense strand and a second polyribonucleotide comprising the antisense strand, and the first caging group is covalently attached to the first polyribonucleotide and to the second polyribonucleotide. For example, the first caging group can be attached to the 5' end of the first polyribonucleotide and to the 3' end of the second polyribonucleotide, or, preferably, it can be attached to the 3' end of the first polyribonucleotide and to the 5' end of the second polyribonucleotide (Figure 4). The caging group linking the two polyribonucleotides can, for example, be photolabile.

[0126] In a preferred aspect, the one or more first caging groups are photoactivatable (e.g., photolabile). Thus, in a preferred class of embodiments, exposing the cell to uncaging energy of the first type comprises exposing the cell to light of a first wavelength (e.g., light with a wavelength between about 60 nm and about 400 nm, between about 400 nm and about 700 nm, and/or between about 700 nm and about 1000 nm). Other caging groups are removable via input of different uncaging energies, e.g., the one or more caging groups can be removable by sonication or application of heat, or can be removed by a chemical or enzyme.

[0127] Exposing the cell to light of a first wavelength optionally comprises exposing the cell to light such that the intensity of the light and the duration of exposure to the light are controlled such that a first portion (which can be a selected amount) of the caged labeled RNA is uncaged and a second portion of the caged labeled RNA remains caged. Put another way, the uncaging rate can be controlled. Furthermore, the uncaging step can be repeated until the caged RNA is depleted.

[0128] As noted, caging permits temporal control over activation of the sensor. For example, the method can include stimulating the cell and uncaging the sensor at a preselected time with respect to the stimulus. For example, the method can include contacting the cell and a test compound and exposing the cell to the uncaging energy at a preselected time point with respect to a time at which the cell and the test compound are contacted (e.g., to determine if the test compound directly or indirectly affects expression of the target mRNA). Caging also permits spatial control over activation of the sensor. For example, the uncaging energy can be directed at a preselected subset of a cell population comprising the cell.

[0129] Various techniques (e.g., lipofection, microinjection, or electroporation) can be used to introduce the labeled RNA into the cell. In one class of embodiments, the labeled RNA also includes a cellular delivery module, associated with the RNA, that can mediate introduction of the labeled RNA into the cell. In this class of embodiments, introducing the labeled RNA into the cell comprises contacting the cell with the labeled RNA associated with the cellular delivery module.

[0130] The cellular delivery module optionally comprises a polypeptide, e.g., a PEP-1 peptide or an amphipathic peptide (e.g., an MPG or an $MPG^{\Delta NLS}$ peptide; see

Simeoni et al. (2003) "Insight into the mechanism of the peptide-based gene delivery system MPG: Implications for delivery of siRNA into mammalian cells" Nucl Acids Res 31: 2717-2724), covalently or noncovalently associated with the RNA. As another example, the polypeptide can be a cationic peptide (e.g., a homopolymer of histidine, lysine, or D-arginine) that is covalently or noncovalently (e.g., by electrostatic interaction with the negatively charged RNA) associated with the RNA. In one class of embodiments, the cellular delivery module comprises a protein transduction domain, e.g., derived from an HIV-1 Tat protein, from a herpes simplex virus VP22 protein, or from a Drosophila antennapedia protein (e.g., Penetratin™). In one aspect, the protein transduction domain is a model protein transduction domain, e.g., a homopolymer of D-arginine, e.g., 8-D-Arg. The protein transduction domain can be covalently attached directly to the RNA, or can be indirectly associated with the RNA (for example, the protein transduction domain can be covalently coupled to a bead or to a carrier protein such as BSA, which is in turn coupled to the RNA, e.g., through a photolabile or cleavable linker; e.g., Figures 8-9).

[0131] The cellular delivery module can be noncovalently associated with the RNA, or the cellular delivery module can be covalently attached to the RNA. For example, the covalent attachment between the cellular delivery module and the RNA is optionally reversible by exposure to light of a preselected wavelength, and the method includes exposing the cell to light of the preselected wavelength. As another example, the covalent attachment is optionally a disulfide bond or an ester linkage that is reduced or cleaved once the sensor is inside the cell.

[0132] In certain embodiments, the cellular delivery module can also serve as a caging group. For example, the RNA can comprise a first polyribonucleotide comprising the sense strand and a second polyribonucleotide comprising the antisense strand, and the cellular delivery module can be covalently attached to the first polyribonucleotide and/or to the second polyribonucleotide (e.g., by a photolabile linker; see, e.g., Figures 7-10). The cellular delivery module can mediate introduction of the RNA into the cell, where the presence of the cellular delivery module prevents the RNA from initiating RNA interference until the cellular delivery module is removed (e.g., by exposing the cell to light of an appropriate wavelength to cleave the photolabile linker).

[0133] Optionally, the cellular delivery module covalently attached to the RNA comprises a lipid, e.g., a fatty acid. For example, the RNA can be covalently attached to one or more myristoyl groups, e.g., via a photolabile linker (Figure 10) .

[0134] In one aspect, the cellular delivery module is associated with one or more second caging groups, which inhibit (e.g., prevent) the cellular delivery module from mediating introduction of the labeled RNA into the cell. In this aspect, the method includes initiating introduction of the labeled RNA into the cell by exposing the labeled RNA to uncaging energy of a second type (which is typically different from the uncaging energy of the first type if first caging groups are present on the RNA), whereby exposure to the uncaging energy frees the cellular delivery module from inhibition by the second caging groups.

[0135] The methods can optionally be used to monitor gene expression, for example, induction of transcription of the target mRNA in response to a stimulus. Thus, in one class of embodiments, the methods include stimulating the cell, e.g., by adding a test compound (e.g., a drug, a candidate drug, a receptor agonist or putative agonist, or the like), by changing growth conditions, by adding other cells, etc.

[0136] The methods can be used to examine expression of the target mRNA, e.g., in two different cell populations, one stimulated and one not. Similarly, expression of the target mRNA can be monitored in a single cell (or a single cell population) before and after stimulation of the cell. Thus, in one embodiment, the signal output is detected at a plurality of time points with respect to a time at which the cell is stimulated.

[0137] The methods optionally include introducing a plurality of RNA sensors (labeled interfering RNAs) into the cell to simultaneously monitor expression of a plurality of target mRNAs. The labels on the different RNAs typically have detectably different signal outputs. For example, the different RNAs can comprise different fluorescent label/quencher combinations (see, e.g., Figure 2) or different donor/acceptor combinations or a combination of FRET, fluorophore/quencher, and TR-FRET sensors can be used (see, e.g., Figure 3). The different sensors are optionally caged, e.g., with photolabile caging groups removable by different wavelengths of light, such that the different sensors can be uncaged at different time points.

[0138] In a related class of embodiments, a reference sensor is also introduced into the cell. Signal output from the target sensor's label (indicating the presence of the target mRNA, e.g., an inducible mRNA, in the cell) can be normalized by comparison with a signal output from the reference sensor. Such a reference sensor can comprise a labeled interfering RNA against a constitutively expressed or housekeeping gene (e.g., GAPDH, actin, or the like).

[0139] In one class of embodiments, the methods are used to determine how efficiently the RNA attenuates (or knocks-down) expression of the target mRNA. In these embodiments, an intensity of the signal output (e.g., intensity of a fluorescent emission) is measured. The intensity provides an indication of the quantity (e.g., relative or absolute quantity) of the target mRNA present in the cell, which provides an indication of the efficiency with which the labeled RNA reduces expression of the target mRNA.

Kits

[0140] Another aspect of the invention includes kits related to the methods. For example, one class of embodiments provides a kit for detecting a target mRNA in a cell. The kit includes a labeled RNA and instructions for using the labeled RNA to detect the presence of the target mRNA in the cell, packaged in one or more containers. The labeled RNA comprises an RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of the target mRNA. The labeled RNA also comprises at least one label, wherein initiation of RNA interference of the target mRNA by the labeled RNA in the cell results in an initiation-dependent change in a signal output of the label.

[0141] The instructions can include, for example, instructions for introducing the labeled RNA into the cell, detecting a fluorescent signal from the RNA, interpreting the fluorescent signal (including quantitating the mRNA based on the intensity of the fluorescent signal), and the like.

[0142] All of the various optional configurations and features noted for the embodiments above apply here as well, to the extent they are relevant, e.g., for label configurations (e.g., use of fluorescent labels, fluorescent label/quencher, and donor/acceptor combinations), signal output types, RNA configurations (e.g., one or two

polyribonucleotides, of various lengths, with or without overhangs, etc.), use of caging groups (e.g., photolabile caging groups), appropriate uncaging energies (light, heat, sonic, etc.), use of cellular delivery modules (e.g., amphipathic peptides, cationic peptides, protein transduction domains, and lipids), and the like.

[0143] In addition, it is worth noting that the kit optionally also includes at least one buffer and/or at least one delivery reagent. The delivery reagent can be essentially any reagent that can mediate introduction of the labeled RNA into the cell; for example, the delivery reagent can comprise a polypeptide (e.g., a PEP-1 peptide, an amphipathic peptide, e.g., an MPG or $MPG^{\Delta NLS}$ peptide, or a cationic peptide, e.g., poly-His, poly-Lys, or poly-D-Arg) or at least one lipid (e.g., a lipid optimized for lipofection of the given labeled RNA, or a lipid comprising at least one myristoyl group to be covalently attached to the labeled RNA). In one class of embodiments, the labeled RNA is caged. In this class of embodiments, the kit optionally includes a control reagent for monitoring uncaging efficiency (e.g., a caged fluorophore, e.g., caged FITC) and/or an uncaged version of the labeled RNA (e.g., to be used as a control to monitor uncaging of the caged labeled RNA, maximal knock down of the target mRNA, and/or the like). The kit also optionally includes packaging or instructional materials for such additional reagents.

[0144] An additional class of embodiments also provides a kit for detecting a target mRNA in a cell. In this class of embodiments, the kit comprises a target RNA sensor and a reference RNA sensor, packaged in one or more containers. The target RNA sensor comprises a first RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA, and at least one first label, wherein initiation of RNA interference of the target mRNA by the first RNA in the cell results in an initiation-dependent change in a signal output of the first label. The reference RNA sensor comprises a second RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a reference mRNA, and at least one second label, wherein initiation of RNA interference of the reference mRNA by the second RNA in the cell results in an initiation-dependent change in a signal output of the second label; packaged in one or more containers.

Typically, the signal output of the first label is detectably different from the signal output of the second label.

[0145] The target and reference mRNAs can be essentially any mRNAs. For example, the target mRNA can be an inducible mRNA while the reference mRNA is a constitutively expressed or housekeeping mRNA (e.g., GAPDH, actin, or the like).

[0146] All of the various optional configurations and features noted for the embodiments above apply here as well, to the extent they are relevant, e.g., for label configurations (e.g., use of fluorescent labels, fluorescent label/quencher, and donor/acceptor combinations), signal output types, RNA configurations (e.g., one or two polyribonucleotides, of various lengths, with or without overhangs, etc.), use of caging groups (e.g., photolabile caging groups), appropriate uncaging energies (light, heat, sonic, etc.), use of cellular delivery modules (e.g., amphipathic peptides, protein transduction domains, and lipids), and the like.

[0147] In addition, it is worth noting that the kit optionally also includes one or more of: instructions (e.g., for using the target and reference RNA sensors to detect the presence of the target mRNA in the cell and/or for using the target and reference RNA sensors to quantitate an amount of the target mRNA present in the cell), a buffer, or a delivery reagent which can mediate introduction of the labeled RNA into the cell (for example, a polypeptide, e.g., a PEP-1 peptide, an amphipathic peptide, e.g., an MPG or $MPG^{\Delta NLS}$ peptide, or a cationic peptide, or at least one lipid, e.g., a lipid optimized for lipofection of the given labeled RNA or a lipid comprising at least one myristoyl group to be covalently attached to the labeled RNA).

Compositions

[0148] Yet another aspect of the invention provides compositions related to the methods (e.g., compositions produced by the methods or facilitating use of the methods). For example, one class of embodiments provides a composition comprising a population of labeled RNAs (e.g., identical labeled RNAs) for detecting a target mRNA in a cell. The target mRNA and/or the cell are also optionally features of the composition. Each labeled RNA comprises an RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of the target mRNA,

and at least one label. The label is located a preselected position in the RNA, and initiation of RNA interference of the target mRNA by the labeled RNA in the cell results in an initiation-dependent change in a signal output of the label.

[0149] All of the various optional configurations and features noted for the embodiments above apply here as well, to the extent they are relevant, e.g., for label configurations (e.g., use of fluorescent labels, fluorescent label/quencher, and donor/acceptor combinations), signal output types, RNA configurations (e.g., one or two polyribonucleotides, of various lengths, with or without overhangs, etc.), use of caging groups (e.g., photolabile caging groups), appropriate uncaging energies (light, heat, sonic, etc.), use of cellular delivery modules (e.g., amphipathic peptides, protein transduction domains, and lipids), and the like. It is worth noting that a quencher or a second label, if present in the RNA, is optionally also located a preselected position in the RNA. The composition comprising the population optionally also includes the target mRNA and/or a cell, e.g., a cell comprising the population and/or the target mRNA.

[0150] Using a population of RNA sensors in which the label is located at a preselected position in the RNA has several advantages over using labeled RNAs where the labels are located at random positions in the RNA. For example, the preselected label position can be chosen such that the initiation-dependent change in the signal output of the label is maximized (e.g., label and quencher or acceptor and donor positions can be selected to maximize the change in signal output). As another example, the preselected label position can be chosen such that the presence of the label does not substantially interfere with initiation of RNAi by the labeled RNA, whereas random labeling of an interfering RNA can result in labels being attached at positions where they interfere with RISC binding to the labeled RNA or the like.

[0151] In another class of embodiments, the invention provides a composition comprising a target RNA sensor and a reference RNA sensor. The target RNA sensor comprises a first RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA, and at least one first label, wherein initiation of RNA interference of the target mRNA by the first RNA in the cell results in an initiation-dependent change in a signal output of the first label. The reference RNA sensor comprises a second RNA comprising at least one double-stranded

region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a reference mRNA, and at least one second label, wherein initiation of RNA interference of the reference mRNA by the second RNA in the cell results in an initiation-dependent change in a signal output of the second label. Typically, the signal output of the first label is detectably different from the signal output of the second label.

[0152] The target and reference mRNAs can be essentially any mRNAs. For example, the target mRNA can be an inducible mRNA while the reference mRNA is a constitutively expressed or housekeeping mRNA (e.g., GAPDH, actin, or the like).

[0153] All of the various optional configurations and features noted for the embodiments above apply here as well, to the extent they are relevant, e.g., for label configurations (e.g., use of fluorescent labels, fluorescent label/quencher, and donor/acceptor combinations), signal output types, RNA configurations (e.g., one or two polyribonucleotides, of various lengths, with or without overhangs, etc.), use of caging groups (e.g., photolabile caging groups), appropriate uncaging energies (light, heat, sonic, etc.), use of cellular delivery modules (e.g., amphipathic peptides, protein transduction domains, and lipids), and the like. It is worth noting that the composition optionally also includes the target mRNA, the reference mRNAs and/or a cell, e.g., a cell comprising the target and reference sensors and/or the target mRNA.

Systems

[0154] In another aspect, systems and/or apparatus comprising the compositions (e.g., the labeled RNAs, cells comprising the labeled RNAs, or the like) noted above and, e.g., components such as detectors, fluid handling apparatus, sources of uncaging energy, or the like, are a feature of the invention.

[0155] In general, various automated systems can be used to perform some or all of the method steps as noted herein. In addition to practicing some or all of the method steps herein, digital or analog systems, e.g., comprising a digital or analog computer, can also control a variety of other functions such as a user viewable display (e.g., to permit viewing of method results by a user) and/or control of output features.

[0156] For example, certain of the methods described above are optionally implemented via a computer program or programs (e.g., that perform or assist in detection

of target mRNA). Thus, the present invention provides digital systems, e.g., computers, computer readable media, and/or integrated systems comprising instructions (e.g., embodied in appropriate software) for performing the methods herein. For example, a digital system comprising instructions for interpreting the change in signal output from the label to determine whether the target mRNA is present in the cell and/or to determine the quantity of the target mRNA present in the cell, as described herein, is a feature of the invention. The digital system can also include information (data) corresponding to signal output intensities or the like. The system can also aid a user in performing mRNA detection according to the methods herein, or can control laboratory equipment which automates introduction of the labeled RNAs into the cells, detection of the signal outputs, or the like.

[0157] Standard desktop applications such as word processing software (e.g., Microsoft Word™ or Corel WordPerfect™) and/or database software (e.g., spreadsheet software such as Microsoft Excel™, Corel Quattro Pro™, or database programs such as Microsoft Access™ or Paradox™) can be adapted to the present invention by inputting data which is loaded into the memory of a digital system and performing an operation as noted herein on the data. For example, systems can include the foregoing software having the appropriate signal intensity (e.g., fluorescent intensity) data, etc., e.g., used in conjunction with a user interface (e.g., a GUI in a standard operating system such as a Windows, Macintosh or LINUX system) to perform any analysis noted herein, or simply to acquire data (e.g., in a spreadsheet) to be used in the methods herein.

[0158] Systems typically include, e.g., a digital computer with software for performing signal output interpretation and/or mRNA quantitation, or the like, as well as data sets entered into the software system comprising signal output intensities or the like. The computer can be, *e.g.*, a PC (Intel x86 or Pentium chip- compatible DOS,™ OS2,™ WINDOWS,™ WINDOWS NT,™ WINDOWS95,™ WINDOWS98,™ LINUX, Apple-compatible, MACINTOSH™ compatible, Power PC compatible, or a UNIX compatible (*e.g.*, SUN™ work station) machine) or other commercially common computer which is known to one of skill. Software for performing analysis of signal output and/or mRNA quantitation can be constructed by one of skill using a standard programming language such as Visualbasic, Fortran, Basic, Java, or the like, according to the methods herein.

[0159] Any system controller or computer optionally includes a monitor which can include, e.g., a cathode ray tube ("CRT") display, a flat panel display (*e.g.*, active matrix

liquid crystal display, liquid crystal display), or others. Computer circuitry is often placed in a box which includes numerous integrated circuit chips, such as a microprocessor, memory, interface circuits, and others. The box also optionally includes a hard disk drive, a floppy disk drive, a high capacity removable drive such as a writeable CD-ROM, and other common peripheral elements. Inputting devices such as a keyboard or mouse optionally provide for input from a user and for user selection of the wavelength of fluorescent emission to be monitored, or the like, in the relevant computer system.

[0160] The computer typically includes appropriate software for receiving user instructions, either in the form of user input into a set parameter fields, *e.g.*, in a GUI, or in the form of preprogrammed instructions, *e.g.*, preprogrammed for a variety of different specific operations. The software then converts these instructions to appropriate language for instructing the system to carry out any desired operation. For example, in addition to performing signal output analysis, a digital system can control laboratory equipment for liquid handling, signal detection, or the like according to the relevant method herein.

[0161] The invention can also be embodied within the circuitry of an application specific integrated circuit (ASIC) or programmable logic device (PLD). In such a case, the invention is embodied in a computer readable descriptor language that can be used to create an ASIC or PLD. The invention can also be embodied within the circuitry or logic processors of a variety of other digital apparatus, such as PDAs, laptop computer systems, displays, image editing equipment, etc.

Applications

[0162] The methods, compositions, systems, and kits described above have a number of applications. For example, as noted, labeled interfering RNA sensors can be used to detect the presence of a target mRNA, to quantitate an amount of a target mRNA present in a cell, and/or to detect activation of a target gene. Additional applications include, but are not limited to, detection of specific biological activities (e.g., GCPR activation, disease, cell migration, cell death, and the like) via detection of activation of biomarker target genes; detection of splice variants of mRNAs, e.g., as biomarkers of biological activities; detection of drug effects via detection of activation of biomarker target genes; and performance of ADME (Absorption, Distribution, Metabolism and Excretion) toxicity assays via detection of activation of biomarker target genes. If desired, high

throughput cell-based assays using labeled interfering RNA sensors can be designed for the above example applications.

CAGED INTERFERING RNA

[0163] In one aspect of this invention, caging groups (e.g., photo-labile caging groups) are used to precisely control the timing and/or location of RNA interference. For example, this invention features photoactivatable (photo activated, PA) interfering RNA sensors suitable for monitoring transcript expression in cells; the sensors are designed for simple operation and are suitable for use in a wide array of instruments (e.g., fluorescent instrument platforms). The caged (e.g., PA) interfering RNAs and methods of use thereof described herein are suitable for applications in, e.g., clinical and basic research and drug discovery.

[0164] The advantages of using a caged (e.g., PA) reaction format include: (1.) controlled activation of reaction components (e.g., controlled initiation of RNAi), (2.) improved assay precision, achieved e.g., (a.) by reducing number of fluidic handling steps in HTS assays – reducing the number of steps (each additional pipetting step can introduce more error into an assay) and/or (b.) by facilitating simultaneous activation of large numbers of assays within millisecond, and (3.) simplified automation and design of miniaturized platforms by reducing the number of steps required. Finally, the caged (e.g., PA) reaction format permits specific activation of a reaction in specific locations – e.g., a subset of wells or locations within a microarray, microfluidic device and/or other miniaturized formats, or even within an organism (e.g., activation of specific locations separated by no more than about a micron is possible).

[0165] An additional advantage of using caged compounds in cells is that caging a molecule frequently renders the molecule more resistant to nucleases, proteases, lipases, and the like, thus extending its half-life in the cell. Caging a molecule which already possesses enhanced resistance to degradation (e.g., by a nuclease or protease, e.g., by incorporation of unnatural amino acids and/or nucleotides into the molecule) offers similar advantages in terms of molecule half-life in the cell or lysate (e.g., thus minimizing false-positive results from undesirable cleavage of a FRET-based sensor or probe).

[0166] Yet another advantage of using caged compounds (e.g., caged interfering RNAs) in cells is that caging a toxic molecule (e.g., an interfering RNA against an essential

gene) frequently protects the cell from the effect of the molecule. This permits compounds that might otherwise be too disruptive to the cell to be utilized in in cell assays; the cell is not subject to the adverse effect of the compound until the compound is uncaged during the assay.

Compositions

[0167] One general class of embodiments provides a composition comprising a caged interfering RNA. The caged RNA includes an RNA having at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. The caged RNA also includes one or more first caging groups associated with the RNA. The first caging groups inhibit (e.g., prevent) the RNA from initiating RNA interference of the target mRNA in a cell comprising the caged RNA.

[0168] The first caging groups can inhibit the RNA from initiating RNA interference of the target mRNA by at least about 25%, at least about 30%, at least about 35%, at least about 50%, at least about 75%, at least about 90%, at least about 95%, or at least about 98%, as compared to the RNA in the absence of the first caging groups. In one class of embodiments, the first caging groups prevent the RNA from initiating RNA interference of the target mRNA (i.e., introduction of the caged RNA into a cell has no effect on expression of the target mRNA). Removal of or an induced conformational change in the first caging groups typically permits the RNA to initiate RNA interference of the target mRNA.

[0169] Note that the first caging groups "inhibiting the RNA from initiating RNA interference" is not meant to imply that the first caging groups inhibit any particular step(s) in the RNAi pathway. For example, the first caging groups can interfere with phosphorylation of a 5' hydroxyl of the antisense strand, RISC binding to the interfering RNA, strand separation of the sense and antisense strands of the interfering RNA, and/or any other step in the RNAi pathway leading to cleavage and degradation of the target mRNA. Initiation of RNAi by the RNA can be indicated, for example, by a decrease in the expression levels of the target mRNA and/or appearance of specific endonucleolytic cleavage fragments of the target mRNA, as is known in the art.

[0170] It is also worth noting that initiation of RNAi of the target mRNA by the RNA need not, but typically does, result in a substantial attenuation of expression of the target mRNA. For example, expression of the target mRNA can be unaffected, or expression of the target mRNA can be decreased by at least about 0.001%, at least about 0.01%, at least about 0.1%, at least about 1%, at least about 5%, at least about 10%, or preferably at least about 25%, at least about 50%, or at least about 75% or more, or can even be reduced to an undetectable level.

[0171] The RNA can have any of a variety of structures, lengths, and/or the like. Thus, in one class of embodiments, the RNA comprises a first polyribonucleotide comprising the sense strand and a second polyribonucleotide comprising the antisense strand. The RNA can be, e.g., a long double-stranded RNA that is cleaved by Dicer in the cell, or it can be, e.g., an siRNA. For example, the first polyribonucleotide can comprise between 19 and 25 nucleotides, the second polyribonucleotide can comprise between 19 and 25 nucleotides, and the double-stranded region can comprise between 19 and 25 base pairs. The first and second polyribonucleotides can form a duplex over their entire length, or they can have overhangs (e.g., 5' or 3' overhangs; e.g., 21 nt first and second polyribonucleotides can form a 19 bp double-stranded region with 2 nucleotide overhangs, 23 nt polyribonucleotides can form a 21 bp double-stranded region with 2 nucleotide overhangs, and so on). For example, in some embodiments, the first polyribonucleotide and the second polyribonucleotide each comprise a two nucleotide TT 3' overhang (where T is 2'-deoxythymidine). The RNA is optionally nuclease resistant and optionally comprises one or more deoxyribonucleotides, one or more PNA monomers, and/or one or more modified nucleotides (e.g., 2'-methyl or 2'-O-allyl ribonucleotides) or internucleotide linkages (e.g., phosphorothioate linkages). In one embodiment, at least one of the one or more first caging groups is covalently attached to a 5' hydroxyl or a 5' phosphate of the second polyribonucleotide. Since this 5' hydroxyl or phosphate is useful for an siRNA to initiate RNAi, caging the 5' hydroxyl or phosphate of the antisense strand permits the sensor to be uncaged and activated in a controlled manner.

[0172] As noted, in certain embodiments, the RNA comprises a first polyribonucleotide comprising the sense strand and a second polyribonucleotide comprising the antisense strand. In other embodiments, the RNA comprises a self-complementary polyribonucleotide (e.g., a hairpin, a shRNA). In either case, the double-stranded region

optionally comprises fewer than about 25 base pairs, fewer than about 30 base pairs, fewer than about 50 base pairs, fewer than about 80 base pairs, fewer than about 150 base pairs, fewer than about 250 base pairs, fewer than about 500 base pairs, fewer than about 1000 base pairs, or fewer than about 1500 base pairs. Although a double-stranded region comprising about 19-25 base pairs is typically sufficient to initiate RNAi, longer regions may be convenient or desirable in certain applications (e.g., double-stranded RNAs longer than 25 bp can stimulate the mammalian immune system, which can be advantageous in certain applications).

[0173] The one or more first caging groups associated with the RNA can be covalently attached to or non-covalently associated with the RNA (e.g., at one or more bases, riboses, phosphate groups and/or terminal hydroxyls, within and/or at the end of either or both strands of the RNA). In one embodiment, the RNA comprises a first polyribonucleotide comprising the sense strand and a second polyribonucleotide comprising the antisense strand, and the first caging group is covalently attached to the first polyribonucleotide and to the second polyribonucleotide. For example, the first caging group can be attached to the 5' end of the first polyribonucleotide and to the 3' end of the second polyribonucleotide, or, preferably, it can be attached to the 3' end of the first polyribonucleotide and to the 5' end of the second polyribonucleotide (Figure 4). The caging group linking the two polyribonucleotides can, for example, be photolabile.

[0174] In a preferred aspect, the one or more first caging groups are photoactivatable (e.g., photolabile). For example, the caging groups can be removed by exposure to light with a wavelength between about 60 nm and about 400 nm, between about 400 nm and about 700 nm, and/or between about 700 nm and about 1000 nm. Other caging groups are removable via input of different uncaging energies; e.g., the one or more caging groups can be removable by sonication or application of heat, or can be removed by a chemical or enzyme.

[0175] In one class of embodiments, the one or more first caging groups each comprises a first binding moiety. The composition also includes a second binding moiety that can bind at least one first binding moiety. For example, the first binding moiety on the caging groups can comprise biotin (see, e.g., Figure 28), and the second binding moiety can comprise avidin or streptavidin. Streptavidin, for example, can thus be bound to the first caging group, increasing its bulkiness and its effectiveness at inhibiting the caged RNA

from participating in RNAi. In some embodiments, the caged RNA comprises two or more first caging groups each comprising the first binding moiety, and the second binding moiety can bind two or more first binding moieties simultaneously. For example, the caged RNA can comprise at least two biotinylated caging groups (e.g., one at the 5' end of the sense strand and one at the 5' end of the antisense strand); binding of streptavidin to multiple biotin moieties on multiple caged RNA molecules links the caged RNAs into a large network. Cleavage of the photolabile group attaching the biotin to the RNA results in dissociation of the network. The uncaged RNA can then participate in RNAi.

[0176] The RNA optionally also includes at least one label, wherein initiation of RNA interference of the target mRNA by the labeled RNA in the cell results in an initiation-dependent change in a signal output of the label. In a preferred class of embodiments, the label is a fluorescent label, and the initiation-dependent change in the signal output of the label is a change in fluorescent emission.

[0177] In one class of embodiments, the labeled RNA also includes at least one quencher. The label and the quencher are positioned in the RNA such that fluorescent emission by the label is quenched by the quencher. Initiation of RNA interference by the labeled RNA results in unquenching of the label (and thus an increase in the fluorescent emission by the label). In this class of embodiments, the initiation-dependent change in the signal output is thus an increase in the fluorescent emission by the label. For example, the label and quencher can be positioned on opposite strands, in close enough proximity to each other that the label is quenched until the sense and antisense strands are separated.

[0178] In one class of embodiments in which the RNA comprises a fluorescent label and a quencher, the RNA comprises a first polyribonucleotide comprising the sense strand and a second polyribonucleotide comprising the antisense strand. The first polyribonucleotide comprises between 19 and 25 nucleotides, the second polyribonucleotide comprises between 19 and 25 nucleotides, and the double-stranded region comprises between 19 and 25 base pairs. The label and quencher can be attached at essentially any suitable positions, e.g., at the 3' end, at the 5' end, and/or within either or both the first and second polyribonucleotides, e.g., as described for the embodiments above. As noted previously, techniques for determining and verifying suitable positions for the label and quencher are well known in the art.

[0179] In a related class of embodiments, the labeled RNA comprises two fluorescent labels, one of which is a donor and the other of which is an acceptor. The donor and acceptor are positioned within the RNA such that energy transfer (e.g., FRET) occurs between them (e.g., excitation of the donor results in fluorescence by the acceptor). Initiation of RNA interference by the labeled RNA results in loss of energy transfer between the donor and the acceptor. This can be observed as an increase in fluorescence by the donor or as a decrease in fluorescence by the acceptor. Thus, in a preferred class of embodiments, the initiation-dependent change in the signal output is a decrease in fluorescent emission by the acceptor following excitation of the donor. For example, the donor and acceptor can be positioned on opposite strands, in close enough proximity to each other that energy transfer occurs until the sense and antisense strands are separated.

[0180] In one class of embodiments in which the RNA comprises a donor and an acceptor, the RNA comprises a first polyribonucleotide comprising the sense strand and a second polyribonucleotide comprising the antisense strand. The first polyribonucleotide comprises between 19 and 25 nucleotides, the second polyribonucleotide comprises between 19 and 25 nucleotides, and the double-stranded region comprises between 19 and 25 base pairs. The donor and acceptor can be attached at essentially any suitable positions, e.g., at the 3' end, at the 5' end, and/or within either or both the first and second polyribonucleotides, e.g., as described for the embodiments above. Techniques for determining and verifying suitable positions for the donor and acceptor are well known in the art.

[0181] In another class of embodiments, the sense strand comprises a first label and the antisense strand a second label. The two labels are different, non-interacting fluorophores with distinct emission spectra (e.g., red and green, such that the double-stranded RNA is yellow while the single strands are red and green).

[0182] The composition optionally also includes the target mRNA and/or a cell, e.g., a cell comprising the caged RNA and/or the target mRNA. Various techniques (e.g., lipofection, microinjection, or electroporation) can be used to introduce the caged RNA into the cell. In one class of embodiments, the caged RNA also includes a cellular delivery module, associated with the RNA, that can mediate introduction of the caged RNA into the cell. All of the various optional configurations and features noted for the embodiments above apply here as well, to the extent they are relevant, e.g., for types of cellular delivery

modules (e.g., polypeptides, amphipathic peptides, protein transduction domains, and lipids), use of one or more second caging groups, and the like.

[0183] Optionally, in the embodiments herein, the caged RNA is bound to a matrix (e.g., electrostatically, covalently, directly or via a linker). In one aspect, the matrix is a surface and the RNA is bound to the surface at a predetermined location within an array comprising other RNAs. In other embodiments, the matrix comprises a bead (e.g., color-coded or otherwise addressable).

[0184] Kits for making the caged RNA (e.g., comprising an RNA, one or more first caging groups, and instructions for assembling the RNA and the first caging groups to form the caged RNA, packaged in one or more containers, and/or one or more first caging groups and instructions for assembling the first caging groups and an RNA supplied by a user of the kit to form the caged RNA, packaged in one or more containers) are also a feature of the invention. Similarly, the invention provides kits for making caged and labeled RNA, e.g., a kit comprising one or more first caging groups, at least one label, and instructions for assembling the first caging groups, at least one label, and an RNA supplied by a user of the kit to form the caged RNA, packaged in one or more containers.

[0185] Kits comprising the caged RNA are another feature of the invention. For example, one class of embodiments provides a kit comprising the caged RNA and one or more of: instructions for using the caged RNA (e.g., to attenuate and/or to detect expression of the target mRNA in a cell), a delivery reagent that can mediate introduction of the caged RNA into a cell, or a buffer, packaged in one or more containers.

[0186] Caging the interfering RNA allows, e.g., precise control over the timing of gene silencing by controlling initiation of RNA interference (also called RNAi or RNA-mediated interference). Use of RNAi for inhibiting gene expression in a number of cell types (including, e.g., mammalian cells) and organisms is well described in the literature, as are methods for determining appropriate interfering RNA(s) to target a desired gene and for generating such interfering RNAs. For example, RNA interference is described e.g., in US patent application publications 20020173478, 20020162126, and 20020182223 and in Hannon G.J. "RNA interference" Nature. 2002 Jul 11;418 (6894):244-51; Ueda R. "RNAi: a new technology in the post-genomic sequencing era" J Neurogenet. 2001;15(3-4):193-204; Ullu et al "RNA interference: advances and questions" Philos Trans R Soc Lond B

Biol Sci. 2002 Jan 29;357(1417):65-70; and Schmid et al "Combinatorial RNAi: a method for evaluating the functions of gene families in Drosophila" Trends Neurosci. 2002 Feb;25 (2):71-4. A kit for producing interfering RNAs is commercially available, e.g., from Ambion, Inc. (www.ambion.com, the Silencer™ siRNA construction kit); kits for randomly labeling such RNAs are available from the same source.

[0187] As noted, single-stranded siRNAs can also initiate RNAi. Thus, another, related general class of embodiments provides a caged interfering RNA. The caged RNA includes an RNA comprising a single polyribonucleotide strand comprising an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. The caged RNA also includes one or more first caging groups associated with the RNA. The first caging groups inhibit (e.g., prevent) the RNA from initiating RNA interference of the target mRNA in a cell comprising the caged RNA. The RNA is typically not self-complementary.

[0188] The single-stranded RNA can have any of a variety of lengths. For example, the polyribonucleotide strand can comprise between 10 and 100 nucleotides, between 10 and 80 nucleotides, between 10 and 50 nucleotides, preferably between 10 and 30 nucleotides, or more preferably between 15 and 30 nucleotides or between 19 and 25 nucleotides.

[0189] The RNA optionally comprises at least one label. In one class of embodiments, initiation of RNA interference of the target mRNA by the labeled RNA in the cell results in an initiation-dependent change in a signal output of the label. For example, the single-stranded RNA can have a fluorescent label at or near one end of the polyribonucleotide and a quencher at or near the other end, or it can have a donor at or near one end of the polyribonucleotide and an acceptor at or near the other end. Alternatively, signal output of the label can be unaffected by participation of the RNA in the RNAi pathway.

[0190] All of the various optional configurations and features noted for the embodiments above apply here as well, to the extent they are relevant, e.g., for label configurations (e.g., use of fluorescent labels, fluorescent label/quencher, and donor/acceptor combinations), signal output types, use of caging groups (e.g., photolabile caging groups), appropriate uncaging energies (light, heat, sonic, etc.), use of cellular

delivery modules (e.g., amphipathic peptides, cationic peptides, protein transduction domains, and lipids), and the like.

Methods

[0191] In one class of methods of the invention, methods of selectively attenuating expression of a target gene in a cell are provided. In the methods, a caged RNA is introduced into the cell. The caged RNA can include an RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA corresponding to the target gene. Alternatively, the caged RNA can include an RNA comprising a single polyribonucleotide strand comprising an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA corresponding to the target gene. The caged RNA comprises one or more caging groups associated with the RNA, the caging groups inhibiting (e.g., preventing) the RNA from initiating RNA interference of the target mRNA in the cell. RNA interference is initiated by exposing the cell to uncaging energy (e.g., light of a predetermined wavelength), freeing the RNA from inhibition by the caging groups.

[0192] Exposing the cell to uncaging energy optionally includes exposing the cell to light of a first wavelength. This exposure can be addressable; e.g., the caged RNA can be exposed to light of the first wavelength by exposing one or more preselected areas (e.g., wells of a microtiter plate or portions thereof, or the like) to the light. As another example, the uncaging energy can be directed at a preselected subset of a cell population comprising the cell.

[0193] Exposing the cell to light of the first wavelength optionally comprises exposing the cell to light such that the intensity of the light and the duration of exposure to the light are controlled such that a first portion (which can be a selected amount) of the caged labeled RNA is uncaged and a second portion of the caged labeled RNA remains caged. Put another way, the uncaging rate can be controlled. Furthermore, the uncaging step can be repeated until the caged RNA is depleted.

[0194] As noted, caging the RNA permits temporal control over initiation of RNA interference. For example, the method can include contacting the cell and a test compound

and exposing the cell to the uncaging energy at a preselected time point with respect to a time at which the cell and the test compound are contacted.

[0195] All of the above optional method variations apply to this method as well. Further, the various composition components noted (particularly the caged RNA embodiments) above can be adapted for use in this method, as appropriate. For example, in one class of embodiments, the caged RNA further comprises a cellular delivery module that can mediate introduction of the caged RNA into the cell, the cellular delivery module being associated with the RNA. In this class of embodiments, the caged RNA is introduced into the cell by contacting the cell with the caged RNA associated with the cellular delivery module. As another example, the cellular delivery module can be covalently attached to the RNA via a photolabile linker, which can be cleaved by exposure to light of an appropriate wavelength once the RNA is inside the cell.

[0196] As another example, in one class of embodiments, the RNA comprises at least one label (e.g., one with an initiation-dependent signal output), and the methods include detecting a signal from the label.

[0197] The methods optionally include introducing a plurality of caged RNAs into the cell. The plurality of caged RNAs can then be uncaged simultaneously or at different times. For example, a first caged RNA can be uncaged, e.g., by exposure to light of a first wavelength, and permitted to initiate RNAi of a first target mRNA. A second caged RNA can be uncaged, e.g., by exposure to light of a second, different wavelength, at a later time.

[0198] In another aspect, systems and/or apparatus comprising the compositions (e.g., the caged RNAs) noted above and, e.g., components such as detectors, fluid handling apparatus, sources of uncaging energy, or the like, are a feature of the invention.

INTERFERING RNAS WITH PROTEIN TRANSDUCTION DOMAINS

[0199] As noted, interfering RNAs can be introduced into cells using protein transduction domains. Thus, one class of embodiments provides a composition comprising an RNA and a protein transduction domain covalently attached to the RNA. The RNA can comprise at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. Alternatively, the RNA can comprise a single polyribonucleotide strand comprising an antisense strand, the antisense

strand comprising a region which is substantially complementary to a region of a target mRNA corresponding to the target gene. The composition optionally also includes the target mRNA and/or a cell, e.g., a cell comprising the caged RNA and/or the target mRNA.

[0200] The protein transduction domain can be essentially any protein transduction domain that can mediate introduction of the RNA into the cell. In one class of embodiments, the protein transduction domain is derived from an HIV-1 Tat protein, from a herpes simplex virus VP22 protein, or from a Drosophila antennapedia protein (e.g., Penetratin™). In other embodiments, the protein transduction domain is a model protein transduction domain, e.g., a homopolymer of lysine, histidine, or D-arginine, e.g., 8-D-Arg.

[0201] The covalent attachment between the protein transduction domain and the RNA is optionally reversible by exposure to light of a preselected wavelength. Similarly, the protein transduction domain can be attached to the RNA through a disulfide bond or an ester linkage that can be reduced or cleaved once the RNA is inside the cell.

[0202] All of the various optional configurations and features noted for the embodiments above apply here as well, to the extent they are relevant, e.g., for RNA configurations (e.g., one or two polyribonucleotides, of various lengths, with or without overhangs, etc.), use of caging groups (e.g., photolabile caging groups), appropriate uncaging energies (light, heat, sonic, etc.), label configurations (e.g., use of fluorescent labels, fluorescent label/quencher, and donor/acceptor combinations), signal output types, binding to a matrix, and the like.

[0203] Kits for making the protein transduction domain-linked RNAs are also a feature of the invention. For example, one embodiment provides a kit comprising an RNA, a protein transduction domain, and instructions for assembling the RNA and the protein transduction domain to form the composition, packaged in one or more containers. A related embodiment provides a kit comprising a protein transduction domain and instructions for assembling the protein transduction domain and an RNA supplied by a user of the kit to form the composition, packaged in one or more containers.

[0204] The invention also provides related methods of introducing an RNA into a cell. In the methods, a composition comprising an RNA and a protein transduction domain covalently attached to the RNA is provided. The RNA can comprise at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense

strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. Alternatively, the RNA can comprise a single polyribonucleotide strand comprising an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA corresponding to the target gene. The composition and the cell are contacted, whereby the protein transduction domain mediates introduction of the RNA into the cell.

[0205] All of the above optional method variations apply to this method as well. Further, the various composition components noted (particularly the protein transduction domain-linked RNA embodiments) above can be adapted for use in this method, as appropriate.

[0206] In another aspect, systems and/or apparatus comprising the compositions noted above and, e.g., components such as detectors, fluid handling apparatus, sources of uncaging energy, or the like, are a feature of the invention.

INTERFERING RNAS WITH LIPIDS

[0207] Interfering RNAs can also be introduced into cells by covalently or non-covalently associated lipids. Thus, one class of embodiments provides a composition comprising an RNA and a lipid covalently or non-covalently attached to the RNA. The RNA can comprise at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA; alternatively, the RNA can comprise a single polyribonucleotide strand comprising an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. The lipid can be, e.g., a fatty acid. In one example class of embodiments, the lipid comprises (or, e.g., consists of) a myristoyl group.

[0208] All of the various optional configurations and features noted for the embodiments above apply here as well, to the extent they are relevant, e.g., for label configurations (e.g., use of fluorescent labels, fluorescent label/quencher, and donor/acceptor combinations), signal output types, RNA configurations (e.g., one or two polyribonucleotides, of various lengths, with or without overhangs, etc.), use of caging groups (e.g., photolabile caging groups), appropriate uncaging energies (light, heat, sonic, etc.), use of cellular delivery modules (e.g., amphipathic peptides, protein transduction

domains, and lipids), and the like. It is worth noting that the composition optionally also includes the target mRNA and/or a cell, e.g., a cell comprising the target mRNA and/or the RNA.

[0209] Kits for making the lipid-linked RNAs are also a feature of the invention. For example, one embodiment provides a kit comprising an RNA, a lipid, and instructions for assembling the RNA and the lipid to form the composition, packaged in one or more containers. A related embodiment provides a kit comprising a lipid and instructions for assembling the lipid and an RNA supplied by a user of the kit to form the composition, packaged in one or more containers.

[0210] The invention also provides related methods of introducing an RNA into a cell. In the methods, a composition comprising an RNA and a lipid covalently attached to the RNA is provided. The RNA can comprise at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA; alternatively, the RNA can comprise a single polyribonucleotide strand comprising an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. The composition and the cell are contacted, whereby the lipid mediates introduction of the RNA into the cell.

[0211] All of the above optional method variations apply to this method as well. Further, the various composition components noted (particularly the lipid-linked RNA embodiments) above can be adapted for use in this method, as appropriate.

[0212] In another aspect, systems and/or apparatus comprising the compositions noted above and, e.g., components such as detectors, fluid handling apparatus, sources of uncaging energy, or the like, are a feature of the invention.

INDUCTION OF INTERFERING RNA EXPRESSION

[0213] In one aspect, the invention includes methods of selectively attenuating expression of a target mRNA in a cell. In the methods, one or more vectors that comprise or encode an RNA are introduced into the cell. The RNA comprises at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of the target mRNA. A caged first activation component is also introduced into the

cell. The caged first activation component includes one or more caging groups associated with a first activation component. The first activation component directly or indirectly increases expression of the RNA from the one or more vectors, and the one or more caging groups inhibit (e.g., prevent) the first activation component from increasing expression of the RNA. The cell is exposed to uncaging energy (e.g., light of a first wavelength), whereby exposure to the uncaging energy frees the first activation component from inhibition by the caging groups. This results in increased expression of the RNA, which can then initiate RNA interference of the target mRNA.

[0214] In one class of embodiments, the first activation component directly increases expression of the RNA from the one or more vectors. For example, the first activation component can be a transcription factor (i.e., a transcriptional activator) or an RNA polymerase, e.g., T7 polymerase.

[0215] In another class of embodiments, the first activation component indirectly increases expression of the RNA by binding to a second activation component, whereby the bound second activation component directly increases expression of the RNA. An example embodiment is schematically illustrated in Figure 30, which depicts tetracycline caged with a photolabile caging group (the caged first activation component). Exposure to light frees the tetracycline from the caging group. In this example, the tetracycline binds a tetracycline-controlled transactivator (tTA, the second activation component), which stimulates transcription of the interfering RNA from a promoter comprising tet operator sequences.

[0216] In yet another class of embodiments, the first activation component indirectly increases expression of the RNA by indirectly activating a third activation component, whereby the activated third activation component directly increases expression of the RNA. An example embodiment is schematically illustrated in Figure 31, which depicts IP3 (inositol 1,4,5-triphosphate) caged with a photolabile caging group (the caged first activation component). Exposure to light frees the IP3 from the caging group, leading to a rise in intracellular Ca^{2+} concentration. The increased Ca^{2+}concentration stimulates calcineurin to dephosphorylate the NF-AT (nuclear factor of activated T cells) transcription factor complex, which then migrates into the nucleus and activates expression of the interfering RNA from a promoter comprising NF-AT-response elements. Caged Ca^{2+}, for example, can also be used as a first activation component in this system.

[0217] Other examples of suitable first activation components include, but are not limited to, cAMP, non-mammalian steroid hormones and small molecules that bind immunophilins. See, e.g., Gossen and Bujard (1992) "Tight control of gene expression in mammalian cells by tetracycline-responsive promoters" Proc. Natl. Acad. Sci. USA 89:5547-5551; Saez et al. (1997) "Inducible gene expression in mammalian cells and transgenic mice" Curr. Opin. Biotechnol. 8:608-616; and Li et al. (1998) "Cell-permeant caged InsP3 ester shows that Ca2+ spike frequency can optimize gene expression" Nature 392:936-541.

[0218] Methods of expressing interfering RNAs of various lengths and structures from vectors are well known in the art. See, e.g., Patterson and Hannon (2002) "Stable suppression of gene expression by RNAi in mammalian cells" Proc. Natl. Acad. Sci. USA 99:1443-1448 and Garbarek and Glover (2003) "RNA interference by production of short hairpin dsRNA in ES cells, their differentiated derivatives, and somatic cell lines" BioTechniques 34:734-744.

[0219] The invention also provides compositions related to the methods. Thus, one general class of embodiments provides a composition comprising one or more vectors and a caged first activation component. The one or more vectors comprise or encode an RNA comprising at least one double-stranded region, the double-stranded region comprising a sense strand and an antisense strand, the antisense strand comprising a region which is substantially complementary to a region of a target mRNA. The caged first activation component comprises one or more caging groups associated with a first activation component, which first activation component directly or indirectly increases expression of the RNA from the one or more vectors in a cell comprising the one or more vectors and the first activation component, and which one or more caging groups inhibit the first activation component from increasing expression of the RNA in the cell. The composition optionally includes the target mRNA and/or a cell, e.g., a cell comprising the one or more vectors and the caged first activation component and/or the target mRNA.

[0220] The composition optionally also includes a second activation component, which second activation component directly increases expression of the RNA when bound by the first activation component (e.g., tetracycline). In a related class of embodiments, the composition optionally also includes a third activation component, which third activation component directly increases expression of the RNA when indirectly activated by the first

activation component. For example, the first activation component can comprise IP3 or Ca^{2+} and the third activation component can comprise an NF-AT transcription factor complex. Other examples of suitable first activation components include, but are not limited to, cAMP, non-mammalian steroid hormones and small molecules that bind immunophilins.

[0221] The length and/or structure of the RNA can vary. For example, the RNA can comprise a first polyribonucleotide comprising the sense strand and a second polyribonucleotide comprising the antisense strand. The double-stranded region formed by annealing of the sense and antisense strands can, e.g., comprise more than about 1500 base pairs, comprise fewer than about 1500 base pairs, fewer than about 1000 base pairs, fewer than about 500 base pairs, fewer than about 250 base pairs, fewer than about 150 base pairs, fewer than about 80 base pairs, fewer than about 50 base pairs, fewer than about 30 base pairs, or even fewer than about 25 base pairs. Instead of comprising a two-stranded interfering RNA (e.g., a siRNA), the RNA comprises a self-complementary polyribonucleotide (e.g., an shRNA).

[0222] Kits form another feature of the invention. Thus, one class of embodiments provides a kit comprising one or more vectors and a caged first activation component, packaged in one or more containers. The kit can also include a vector that comprises or encodes a second or a third activation component, and/or instructions for using the kit, e.g., instructions for using the kit to attenuate expression of a target mRNA.

[0223] All of the various optional configurations and features noted for the embodiments above apply to the methods and compositions here as well, to the extent they are relevant, e.g., RNA configurations (e.g., one or two polyribonucleotides, of various lengths, with or without overhangs, etc.), use of caging groups (e.g., photolabile caging groups), appropriate uncaging energies (light, heat, sonic, etc.), use of cellular delivery modules (e.g., amphipathic peptides, protein transduction domains, and lipids), and the like.

CAGED DNAS ENCODING INTERFERING RNAS

[0224] The invention also includes other methods of selectively attenuating expression of a target gene in a cell. In the methods, a first caged DNA and a second caged DNA are introduced into the cell. The first caged DNA includes a first DNA encoding an RNA sense strand and one or more caging groups. The second caged DNA comprises a

second DNA encoding an RNA antisense strand and one or more caging groups. The presence of the caging groups prevents transcription of the first and second DNAs, the first and second DNAs each comprising at least a portion of the target gene, and the sense and antisense strands being at least partially complementary and able to form a duplex over at least a portion of their lengths. RNA interference is initiated by generating double-stranded RNA by exposing the cell to uncaging energy, whereby exposure to the uncaging energy frees the first and second DNAs from the caging groups and permits transcription of the first and second DNAs to occur.

[0225] The resulting double-stranded RNA can comprise two distinct polyribonucleotides (i.e., the sense strand can comprise a first polyribonucleotide while the antisense strand comprises a second polyribonucleotide), or the resulting double-stranded RNA can comprise a single, self-complementary polyribonucleotide that includes the sense and antisense strands (e.g., an shRNA).

[0226] All of the above optional method variations apply to this method as well, to the extent they are relevant. Further, the various composition components noted above can be adapted for use in this method, as appropriate; e.g., use of caging groups (e.g., photolabile caging groups), appropriate uncaging energies (light, heat, sonic, etc.), use of cellular delivery modules (e.g., amphipathic peptides, protein transduction domains, and lipids), and the like. It is worth noting that when the resulting double-stranded RNA comprises a single, self-complementary polyribonucleotide, the first and second DNAs are covalently joined in proximity to each other as a single transcription unit, e.g., on a plasmid. When the resulting double-stranded RNA comprises two distinct polyribonucleotides, the first and second DNAs can be on separate plasmids or can optionally be included on a single plasmid (see, e.g., US patent application publication 20020182223). The DNAs are optionally nuclease resistant.

CAGING GROUPS

[0227] A large number of caging groups, and a number of reactive compounds that can be used to covalently attach caging groups to other molecules, are well known in the art. Examples of photolabile caging groups include, but are not limited to: 2-nitrobenzyl; 1-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE); brominated 7-hydroxycoumarin-4-ylmethyls (e.g., 6-Bromo-7-hydroxycoumarin-4-ylmethyl (Bhc)); nitroindolines; N-acyl-7-

nitroindolines; phenacyls; hydroxyphenacyl; benzoin esters; dimethoxybenzoin; meta-phenols; 4,5-dimethoxy-2-nitrobenzyl (DMNB); alpha-carboxy-2-nitrobenzyl (CNB); 1-(2-nitrophenyl)ethyl (NPE); 5-carboxymethoxy-2-nitrobenzyl (CMNB); (5-carboxymethoxy-2-nitrobenzyl)oxy) carbonyl; (4,5-dimethoxy-2-nitrobenzyl)oxy) carbonyl; desoxybenzoinyl; and the like. See e.g., USPN 5,635,608 to Haugland and Gee (June 3, 1997) entitled "α-carboxy caged compounds"; Neuro 19, 465 (1997); J Physiol 508.3, 801 (1998); Proc Natl Acad Sci USA 1988 Sep; 85(17):6571-5; J Biol Chem 1997 Feb 14; 272(7):4172-8; Neuron 20,619-624, 1998; Nature Genetics, vol. 28:2001:317-325; Nature, vol. 392,1998:936-941; Pan, P., and Bayley,, H. "Caged cysteine and thiophosphoryl peptides" FEBS Letters 405:81-85 (1997); Pettit et al. (1997) "Chemical two-photon uncaging: a novel approach to mapping glutamate receptors" Neuron 19:465-471; Furuta et al. (1999) "Brominated 7-hydroxycoumarin-4-ylmethyls: novel photolabile protecting groups with biologically useful cross-sections for two photon photolysis" Proc. Natl. Acad. Sci. 96(4):1193-1200; Zou et al. "Catalytic subunit of protein kinase A caged at the activating phosphothreonine" J. Amer. Chem. Soc. (2002) 124: 8220-8229; Zou et al. "Caged Thiophosphotyrosine Peptides" Angew. Chem. Int. Ed. (2001) 40: 3049-3051; Conrad II et al. "p-Hydroxyphenacyl Phototriggers: The Reactive Excited State of Phosphate Photorelease" J. Am. Chem. Soc. (2000) 122:9346-9347; Conrad II et al. "New Phototriggers 10: Extending the π,π^* Absorption to Release Peptides in Biological Media" Org. Lett. (2000) 2:1545-1547; Givens et al. "A New Phototriggers 9: p-Hydroxyphenacyl as a C-Terminus Photoremovable Protecting Group for Oligopeptides" J. Am. Chem. Soc. (2000) 122:2687-2697; Bishop et al. "40-Aminomethyl-2,20-bipyridyl-4-carboxylic Acid (Abc) and Related Derivatives: Novel Bipyridine Amino Acids for the Solid-Phase Incorporation of a Metal Coordination Site Within a Peptide Backbone" Tetrahedron (2000)56:4629-4638; Ching et al "Polymers As Surface-Based Tethers with Photolytic triggers Enabling Laser-Induced Release/Desorption of Covalently Bound Molecules" Bioconjugate Chemistry (1996) 7:525-8; USPN 5,888,829 to Gee and Millard (March 30, 1999) entitled "Photolabile caged ionophores and method of using in a membrane separation process"; USPN 6,043,065 to Kao et al. (March 28, 2000) entitled "Photosensitive organic compounds that release 2,5,-di(tert-butyl) hydroquinone upon illumination"; USPN 5,430,175 to Hess et al. (July 4, 1995) entitled "Caged carboxyl compounds and use thereof"; USPN 5872243; PNAS (1980) 77:7237-41; BioProbes Handbook, 2002 from Molecular Probes, Inc.; and

Handbook of Fluorescent Probes and Research Products, Ninth Edition or Web Edition, from Molecular Probes, Inc, as well as the references below. Many compounds, kits, etc. for use in caging various molecules are commercially available, e.g., from Molecular Probes, Inc. (www.molecularprobes.com).

[0228] Environmentally responsive polymers suitable for use as caging groups have also been described. Such polymers undergo conformational changes induced by light, an electric or magnetic field, a change in pH and/or ionic strength, temperature, or addition of an antigen or saccharide, or other environmental variables. For example, Shimoboji et al. (2002) "Photoresponsive polymer-enzyme switches" Proc. Natl. Acad. Sci. USA 99:16,592-16,596 describes polymers that undergo reversible conformational changes in response to light; such polymers can, e.g., be used as photoactivatable caging groups. See also Ding et al. (2001) "Size-dependent control of the binding of biotinylated proteins to streptavidin using a polymer shield" Nature 411:59-62; Miyata et al. (1999) "A reversibly antigen-responsive hydrogel" Nature 399:766-769; Murthy et al. (2003) "Bioinspired pH-responsive polymers for the intracellular delivery of biomolecular drugs" Bioconjugate Chem. 14:412-419; and Galaev and Mattiasson (1999) "'Smart' polymers and what they could do in biotechnology and medicine" Trends Biotech. 17:335-340. Figures 26 and 27 schematically illustrate use of environmentally responsive polymers as caging groups. Figure 26 illustrates noncovalent association of a polymer with a component to be caged (e.g., an siRNA). In its folded conformation, the polymer physically surrounds and traps the component (Panel B). The caged RNA is optionally introduced into a cell. A conformational change in the polymer induced by light, pH, temperature, or the like results in release of the RNA from the unfolded conformation of the polymer (Panel D). Figure 27 illustrates covalent association of a polymer with an example double-stranded siRNA. In its folded conformation, the polymer prevents the siRNA from initiating RNAi (e.g., by preventing the siRNA from interacting with a kinase, RISC, or other components of the RNAi cellular machinery) (Panel A). A conformational change in the polymer induced by light, pH, temperature, or the like permits the siRNA to initiate RNAi (Panel B).

[0229] Caged polypeptides (including, e.g., polypeptide cellular delivery modules, e.g., protein transduction domains) can be produced, for example, by reacting a polypeptide with a caging compound or by incorporating a caged amino acid during synthesis of a polypeptide. See, e.g., USPN 5,998,580 to Fay et al. (December 7, 1999) entitled

"Photosensitive caged macromolecules"; Kossel et al. (2001) PNAS 98:14702-14707; Trends Plant Sci (1999) 4:330-334; PNAS (1998) 95:1568-1573; J Am Chem Soc (2002) 124:8220-8229; Pharmacology & Therapeutics (2001) 91:85-92; and Angew Chem Int Ed Engl (2001) 40:3049-3051. A photolabile polypeptide linker (e.g., for connecting a protein transduction domain and an RNA, or the like) can, for example, comprise a photolabile amino acid such as that described in USPN 5,998,580 (supra).

[0230] Caged nucleic acids (e.g., DNA, RNA or PNA, e.g., interfering RNAs) can be produced by reacting the nucleic acids with caging compounds or by incorporating a caged nucleotide during synthesis of a nucleic acid. For example, USPN 6,242,258 to Haselton and Alexander (June 5, 2001) entitled "Methods for the selective regulation of DNA and RNA transcription and translation by photoactivation" and USPN 6,410,327 to Haselton, III, et al. entitled "Methods for the selective regulation of DNA and RNA transcription and translation by photoactivation" describe DMNPE caging of DNA by postsynthetic reactions; Ando et al. (2001) "Photo-mediated gene activation using caged RNA/DNA in zebrafish embryos" Nature Genetics 28: 317-325 describes Bhc caging of RNA and DNA by postsynthetic reactions; and Chaulk and MacMillan (1998) "Caged RNA: Photo-control of a ribozyme reaction" Nucl Acids Res. 26:3173-3178 describes 2-nitrobenzyl caging of RNA by incorporation of a caged phosphoramidite during RNA synthesis. A caged RNA or an RNA that is to be caged optionally includes one or more deoxyribonucleotides and/or nonnatural or modified nucleotides, e.g., that are less reactive than standard ribonucleotides, to facilitate attachment of the caging group(s), e.g., to a 5' hydroxyl.

[0231] Caging groups can be attached at random and/or predetermined sites within a molecule. Useful site(s) of attachment of and/or conditions for attaching caging groups to a given molecule can be determined by techniques known in the art. For example, a molecule with a known activity (e.g., an interfering RNA or a protein transduction domain) can be reacted with a caging compound. The resulting caged molecule can then be tested to determine if its activity (e.g., ability to initiate RNAi or to mediate introduction of an associated molecule into a cell) is sufficiently abrogated. As another example, amino acid residues central to the activity of a polypeptide (e.g., residues located at a binding interface of a protein transduction domain, or the like) can be identified by routine techniques such as scanning mutagenesis, sequence comparisons and site-directed mutagenesis, or the like.

Such residues can then be caged, and the activity of the caged polypeptide (e.g., its ability to mediate introduction of an associated molecule into a cell) can be assayed to determine the efficacy of caging. Similarly, an RNA can be caged at positions and/or groups identified as being required for activity (e.g., the 5' phosphate or 5' hydroxyl of the antisense strand of an siRNA can be caged).

[0232] An alternative method for caging a molecule (e.g., an siRNA) is to enclose the molecule in a photolabile vesicle (e.g., a photolabile lipid vesicle), optionally including a protein transduction domain or the like (Figure 11). Similarly, the molecule can be loaded into the pores of a porous bead which is then encased in a photolabile gel.

[0233] Appropriate methods for uncaging caged molecules are also known in the art. For example, appropriate wavelengths of light for removing many photolabile groups have been described; e.g., 300-360 nm for 2-nitrobenzyl, 350 nm for benzoin esters, and 740 nm for brominated 7-hydroxycoumarin-4-ylmethyls (two-photon) (see, e.g., references herein). Conditions for uncaging any caged molecule (e.g., the optimal wavelength for removing a photolabile caging group) can be determined according to methods well known in the art. Instrumentation and devices for delivering uncaging energy are likewise known (e.g., sonicators, heat sources, light sources, and the like). For example, well known and useful light sources include e.g., a lamp, a laser (e.g., a laser optically coupled to a fiber-optic delivery system) or a light-emitting compound.

IN VIVO AND IN VITRO CELLULAR DELIVERY

[0234] Molecules (e.g., double-stranded RNAs, including caged and/or labeled RNAs) can be introduced into cells by traditional methods such as lipofection, electroporation, microinjection, optofection, laser transfection, calcium phosphate precipitation, and/or particle bombardment. Double-stranded RNA can also be introduced into cells by pinocytosis or by using streptolysin-O (SLO). See, e.g., WO 03/040375 by Wolff entitled "Compositions and processes using siRNA, amphipathic compounds and polycations." Reagents for delivery of double-stranded RNAs are commercially available, e.g., *Trans*IT-TKO™ (Mirus Corporation, www.genetransfer.com). If the molecule is caged, such delivery can be accomplished without uncaging and thereby activating the molecule; for example, a photoactivatable interfering RNA is not active during the delivery process until exposed to light of appropriate wavelength. However, these methods require

manipulation of the cells, e.g., adding and removing transfection materials, pre-treating cells, and special apparatus and equipment, etc. In addition, some cells (particularly primary cells) are difficult to transfect by methods such as lipofection.

[0235] While the methods above are suitable for introducing molecules (e.g., interfering RNAs and caged DNAs) into cells, this invention features a simpler and more effective method of introducing molecules into the cell. That is, the molecule is optionally associated (covalently or non-covalently) with a cellular delivery module that can mediate its introduction into the cell. The cellular delivery module is typically, but need not be, a polypeptide, for example, a PEP-1 peptide, an amphipathic peptide, e.g., an MPG peptide (Simeoni et al. (2003) "Insight into the mechanism of the peptide-based gene delivery system MPG: Implications for delivery of siRNA into mammalian cells" Nucl Acids Res 31: 2717-2724), a cationic peptide (e.g., a homopolymer of lysine, histidine, or D-arginine), or a protein transduction domain (a polypeptide that can mediate introduction of a covalently associated molecule into a cell). See, e.g., Lane (2001) Bioconju Chem., 12:825-841; Bonetta (2002) The Scientist 16:38; and Curr Opin Mol Ther (2000) 2:162-7. For example, an interfering RNA (including a caged and/or labeled interfering RNA) can be covalently associated with a protein transduction domain (e.g., an HIV TAT sequence, which most cells naturally uptake, or a short D-arginine homopolymer, e.g., 8-D-Arg, eight contiguous D-arginine residues). The protein transduction domain-coupled RNA can simply be, e.g., added to cell culture or injected into an animal for delivery. (Note that TAT and D- arginine homopolymers, for example, can alternatively be noncovalently associated with the interfering RNA and still mediate its introduction into the cell.)

[0236] A number of polypeptides capable of mediating introduction of associated molecules into a cell are known in the art and can be adapted to the present invention; see, e.g., the references above and Langel (2002) Cell Penetrating Peptides CRC Press, Pharmacology & Toxicology Series.

[0237] As noted, an interfering RNA, or a caged DNA, can also be introduced into cells by covalently or noncovalently attached lipids, e.g., by a covalently attached myristoyl group. In any of the cellular delivery modules herein, lipids used for lipofection are optionally excluded from cellular delivery modules in some embodiments.

[0238] In summary, an interfering RNA or a caged DNA can be introduced into a cell by any of several methods, including without limitation, lipofection, electroporation, microinjection, and association with a cellular delivery module (including covalent association with a protein transduction domain). Interfering RNA and caged DNA can optionally be introduced into specific tissues and/or cell types (e.g., explanted or in an organism), for example, by laser transfection, gold particle bombardment, microinjection, coupling to viral proteins, or covalent association with a protein transduction domain, among other techniques. See, e.g., Robbins et al. (2002) "Peptide delivery to tissues via reversibly linked protein transduction sequences" Biotechniques 33:190-192 and Rehman et al. (2003) "Protection of islets by in situ peptide-mediated transduction of the I-kappa B kinase inhibitor Nemo-binding domain peptide" J Biol Chem 278:9862-9868.

[0239] The cell into which an interfering RNA or a caged DNA of this invention is introduced is typically a eukaryotic cell (e.g., a yeast, a vertebrate cell, a mammalian cell, a rodent cell, a primate cell, a human cell, a plant cell, an insect cell, or essentially any other type of eukaryotic cell). The cell can be, e.g., in culture or in a tissue, fluid, etc. and/or from or in an organism.

[0240] The cellular delivery modules optionally can be caged. Covalently associated cellular delivery modules (e.g., protein transduction domains) can optionally be released from the associated molecule (e.g., by placement of a photolabile linkage, a disulfide or ester linkage that is reduced or cleaved in the cell, or the like, between the cellular delivery module and the molecule). For example, 8-D-Arg can be covalently linked through a disulfide linker to an interfering RNA. The 8-D-Arg module mediates entry of the RNA into a cell, where the linker is reduced in the reducing environment of the cytoplasm, freeing the interfering RNA from the 8-D-Arg module.

[0241] The amount of a nucleic acid delivered to a cell can optionally be controlled by controlling the number of cellular delivery modules associated with the nucleic acid (covalently or noncovalently). For example, increasing the ratio of 8-D-Arg to interfering RNA can increase the percentage of interfering RNA that enters the cell.

[0242] The interfering RNAs and caged DNAs of this invention optionally also comprise a subcellular delivery module (e.g., a peptide, nucleic acid, and/or carbohydrate tag) or other means of achieving a desired subcellular localization. For example, an

interfering RNA is typically most effective at initiating RNAi when it is localized to the cytoplasm. Thus, if a method that results in localization of the interfering RNA to the endosome is used to introduce the RNA into the cell (e.g., lipofection, certain protein transduction domains, and the like), performance of the interfering RNA can be improved by including an endosomal release agent on the RNA (e.g., HA-2, PEI, or a dendrimer). See, e.g., Journal of Controlled Release (1999) 61:137-143; J Biol Chem 277:27135-43; Proc Natl Acad Sci 89:7934-38; and Bioconjugate Chem (2002) 13:996-1001. Examples of subcellular delivery modules include nuclear localization signals, chloroplast stromal targeting sequences, and many others (see, e.g., Molecular Biology of the Cell (3rd ed.) Alberts et al., Garland Publishing, 1994; and Molecular Cell Biology (4th ed.) Lodish et al., W H Freeman & Co, 1999). Similarly, localization can be to a target protein; that is, the subcellular delivery module can comprise a binding domain that binds the target protein.

LABELS

[0243] The compositions of this invention optionally include one or more labels; e.g., optically detectable labels, such as fluorescent or luminescent labels, and/or non optically detectable labels, such as magnetic labels. A number of fluorescent labels are well known in the art, including but not limited to, quantum dots, hydrophobic fluorophores (e.g., coumarin, rhodamine and fluorescein), and green fluorescent protein (GFP) and variants thereof (e.g., cyan fluorescent protein and yellow fluorescent protein). See e.g., Haughland (2002) Handbook of Fluorescent Probes and Research Products, Ninth Edition or the current Web Edition, both available from Molecular Probes, Inc. Likewise, a variety of donor/acceptor and fluorophore/quencher combinations, using e.g., fluorescence resonance energy transfer (FRET)-based quenching, non-FRET based quenching, or wavelength-shifting harvester molecules, are known. Example combinations include cyan fluorescent protein and yellow fluorescent protein, terbium chelate and TRITC (tetrarhodamine isothiocyanate), lanthanide (e.g., europium or terbium) chelates and allophycocyanin (APC) or Cy5, europium cryptate and Allophycocyanin, fluorescein and tetramethylrhodamine, IAEDANS and fluorescein, EDANS and DABCYL, fluorescein and DABCYL, fluorescein and fluorescein, BODIPY FL and BODIPY FL, and fluorescein and QSY 7 dye. Nonfluorescent acceptors such as DABCYL and QSY 7 and QSY 33 dyes have the particular advantage of eliminating background fluorescence resulting from direct

(i.e., nonsensitized) acceptor excitation. See, e.g., U.S. Pat. Nos. 5,668,648, 5,707,804, 5,728,528, 5,853,992, and 5,869,255 to Mathies et al. for a description of FRET dyes.

[0244] For use of quantum dots as labels for biomolecules, see e.g., Dubertret et al. (2002) Science 298:1759; Nature Biotechnology (2003) 21:41-46; and Nature Biotechnology (2003) 21:47-51. In the context of the present invention, such quantum dots can be used to label any nucleic acid of interest, e.g., an interfering RNA, e.g., a caged interfering RNA.

[0245] Other optically detectable labels can also be used in the invention. For example, gold beads can be used as labels and can be detected using a white light source via resonance light scattering. See, e.g., http://www.geniconsciences.com. Suitable non-optically detectable labels are also known in the art. For example, magnetic labels can be used in the invention (e.g., 3 nm superparamagnetic colloidal iron oxide as a label and NMR detection; see e.g., Nature Biotechnology (2002) 20:816-820).

[0246] Labels can be introduced to nucleic acids during synthesis or by postsynthetic reactions by techniques established in the art. For example, a fluorescently labeled nucleotide can be incorporated into an RNA or DNA during enzymatic or chemical synthesis of the nucleic acid, e.g., at a preselected or random nucleotide position. Alternatively, fluorescent labels can be added to RNAs or DNAs by postsynthetic reactions, at either random or preselected positions (e.g., an oligonucleotide can be chemically synthesized with a terminal amine or free thiol at a preselected position, and a fluorophore can be coupled to the oligonucleotide via reaction with the amine or thiol). Reagents for fluorescent labeling of nucleic acids are commercially available; for example, a variety of kits for fluorescently labeling nucleic acids are available from Molecular Probes, Inc. (www.probes.com), and a kit for randomly labeling double-stranded RNA is available from Ambion, Inc. (www.ambion.com, the Silencer™ siRNA labeling kit). Quenchers can be introduced by analogous techniques.

[0247] Attachment of labels to oligos during automated synthesis and by post-synthetic reactions has been described. See, e.g., Tyagi and Kramer (1996) "Molecular beacons: probes that fluoresce upon hybridization" Nature Biotechnology 14:303-308; USPN 6,037,130 to Tyagi et al. (March 14, 2000), entitled "Wavelength-shifting probes and primers and their use in assays and kits"; and USPN 5,925,517 (July 20, 1999) to Tyagi et

al. entitled "Detectably labeled dual conformation oligonucleotide probes, assays and kits." Additional details on synthesis of functionalized oligos can be found in Nelson, et al. (1989) "Bifunctional Oligonucleotide Probes Synthesized Using A Novel CPG Support Are Able To Detect Single Base Pair Mutations" Nucleic Acids Research 17:7187-7194.

[0248] Labels and/or quenchers can be introduced to the oligonucleotides, for example, by using a controlled-pore glass column to introduce, e.g., the quencher (e.g., a 4-dimethylaminoazobenzene-4'-sulfonyl moiety (DABSYL). For example, the quencher can be added at the 3' end of oligonucleotides during automated synthesis; a succinimidyl ester of 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL) can be used when the site of attachment is a primary amino group; and 4-dimethylaminophenylazophenyl-4'-maleimide (DABMI) can be used when the site of attachment is a sulphydryl group. Similarly, fluorescein can be introduced into oligos, either using a fluorescein phosphoramadite that replaces a nucleoside with fluorescein, or by using a fluorescein dT phosphoramadite that introduces a fluorescein moiety at a thymidine ring via a spacer. To link a fluorescein moiety to a terminal location, iodoacetoamidofluorescein can be coupled to a sulphydryl group. Tetrachlorofluorescein (TET) can be introduced during automated synthesis using a 5'-tetrachloro-fluorescein phosphoramadite. Other reactive fluorophore derivatives and their respective sites of attachment include the succinimidyl ester of 5-carboxyrhodamine-6G (RHD) coupled to an amino group; an iodoacetamide of tetramethylrhodamine coupled to a sulphydryl group; an isothiocyanate of tetramethylrhodamine coupled to an amino group; or a sulfonylchloride of Texas red coupled to a sulphydryl group. Labeled oligonucleotides can be purified, if desired, e.g., by high pressure liquid chromatography or other methods.

[0249] Similarly, signals from the labels (e.g., absorption by and/or fluorescent emission from a fluorescent label) can be detected by essentially any method known in the art. For example, multicolor detection, detection of FRET (including, e.g., time-resolved or TR-FRET, e.g., between lanthanide chelate donors and fluorescent dye acceptors; see, e.g., Journal of Biomolecular Screening (2002) 7:3-10), and the like, are well known in the art. In brief, FRET (Fluorescence Resonance Energy Transfer) is a non-radiative energy transfer phenomenon in which two fluorophores with overlapping emission and excitation spectra, when in sufficiently close proximity, experience energy transfer by a resonance dipole induced dipole interaction. The phenomenon is commonly used to study the binding

of analytes such as nucleic acids, proteins and the like. FRET is a distance dependent excited state interaction in which emission of one fluorophore is coupled to the excitation of another which is in proximity (close enough for an observable change in emissions to occur). Some excited fluorophores interact to form excimers, which are excited state dimers that exhibit altered emission spectra (e.g., phospholipid analogs with pyrene sn-2 acyl chains); *see, e.g.,* Haughland (2003) Handbook of Fluorescent Probes and Research Products Ninth Edition, available from Molecular Probes. A straightforward discussion of FRET can be found in the Handbook and the references cited therein.

[0250] As another example, fluorescence polarization can be used. Briefly, in the performance of such fluorescent binding assays, a typically small, fluorescently labeled molecule, e.g., a ligand, antigen, etc., having a relatively fast rotational correlation time, is used to bind to a much larger molecule, e.g., a receptor protein, antibody etc., which has a much slower rotational correlation time. The binding of the small labeled molecule to the larger molecule significantly increases the rotational correlation time (decreases the amount of rotation) of the labeled species, namely the labeled complex over that of the free unbound labeled molecule. This has a corresponding effect on the level of polarization that is detectable. Specifically, the labeled complex presents much higher fluorescence polarization than the unbound, labeled molecule.

[0251] Generally, fluorescence polarization level is calculated using the following formula:

$$P=[I_1-I_2]/[I_1+I_2]$$

where I_1 is the fluorescence detected in the plane parallel to the excitation light, and I_2 is the fluorescence detected in the plane perpendicular to the excitation light. References which discuss fluorescence polarization and/or its use in molecular biology include Perrin (1926) "Polarization de la lumiere de fluorescence. Vie moyenne de molecules dans l'etat excite" J Phys Radium 7:390; Weber (1953) "Rotational Brownian motion and polarization of the fluorescence of solutions" Adv Protein Chem 8:415; Weber (1956) J Opt Soc Am 46:962; Dandliker and Feigen (1961) "Quantification of the antigen-antibody reaction by the polarization of fluorescence" Biochem Biophys Res Commun 5:299; Dandliker and de Saussure (1970) "Fluorescence polarization in immunochemistry" Immunochemistry 7:799; Dandliker et al. (1973) "Fluorescence polarization immunoassay. Theory and experimental

method" Immunochemistry 10:219; Levison et al. (1976) "Fluorescence polarization measurement of the hormone-binding site interaction" Endocrinology 99:1129; Jiskoot et al. (1991) "Preparation and application of a fluorescein-labeled peptide for determining the affinity constant of a monoclonal antibody-hapten complex by fluorescence polarization" Anal Biochem 196:421; Wei and Herron (1993) "Use of synthetic peptides as tracer antigens in fluorescence polarization immunoassays of high molecular weight analytes" Anal Chem 65:3372; Devlin et al. (1993) "Homogeneous detection of nucleic acids by transient-state polarized fluorescence" Clin Chem 39:1939; Murakami et al. (1991) Fluorescent-labeled oligonucleotide probes detection of hybrid formation in solution by fluorescence polarization spectroscopy" Nuc. Acids Res 19:4097; Checovich et al. (1995) "Fluorescence polarization-a new tool for cell and molecular biology" Nature 375:354-256; Kumke et al. (1995) "Hybridization of fluorescein-labeled DNA oligomers detected by fluorescence anisotropy with protein binding enhancement" Anal Chem 67:21, 3945-3951; and Walker et al. (1996) "Strand displacement amplification (SDA) and transient-state fluorescence polarization detection of mycobacterium tuberculosis DNA" Clinical Chemistry 42:1, 9-13.

ARRAYS

[0252] In certain embodiments, the RNA is arranged in an array. In an array on a matrix (e.g., a surface), each nucleic acid is bound (e.g., electrostatically or covalently bound, directly or via a linker) to the matrix at a unique location. Methods of making, using, and analyzing such arrays (e.g., microarrays) are well known in the art, including methods of using arrays by overlaying the arrays with cells into which the components of the array can be introduced. See e.g., USPN 6,197,599; Ziauddin and Sabatini "Microarrays of cells expressing defined cDNAs" Nature 2001 May 3;411(6833):107-10; and Falsey et al. Bioconjug. Chem. (2001) 12:346-53.

MOLECULAR BIOLOGICAL TECHNIQUES

[0253] In practicing the present invention, many conventional techniques in molecular biology, microbiology, and recombinant DNA technology are optionally used (e.g., for making and/or manipulating nucleic acids, polypeptides, and/or cells of the invention). These techniques are well known, and detailed protocols for numerous such procedures (including, e.g., in vitro amplification of nucleic acids, cloning, mutagenesis,

transformation, cellular transduction with nucleic acids, protein expression, and/or the like) are described in, for example, Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology volume 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook et al., Molecular Cloning - A Laboratory Manual (3rd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 2002 ("Sambrook") and Current Protocols in Molecular Biology, F.M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (supplemented through 2002) ("Ausubel")). Other useful references, e.g. for cell isolation and culture (e.g., for subsequent nucleic acid or protein isolation) include Freshney (1994) Culture of Animal Cells, a Manual of Basic Technique, third edition, Wiley- Liss, New York and the references cited therein; Payne *et al.* (1992) Plant Cell and Tissue Culture in Liquid Systems John Wiley & Sons, Inc. New York, NY; Gamborg and Phillips (Eds.) (1995) Plant Cell, Tissue and Organ Culture; Fundamental Methods Springer Lab Manual, Springer-Verlag (Berlin Heidelberg New York) and Atlas and Parks (Eds.) The Handbook of Microbiological Media (1993) CRC Press, Boca Raton, FL.

Oligonucleotide Synthesis

[0254] In general, synthetic methods for making oligonucleotides and PNAs (including labeled oligos and PNAs) is well known. For example, oligonucleotides can be synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage and Caruthers (1981), Tetrahedron Letts., 22(20):1859-1862, e.g., using a commercially available automated synthesizer, e.g., as described in Needham-VanDevanter et al. (1984) Nucleic Acids Res., 12:6159-6168. Synthesis of PNAs and modified oligonucleotides (e.g., oligonucleotides comprising 2'-O-methyl nucleotides and/or phosphorothioate, methylphosphonate, or boranophosphate linkages) are described in e.g., Oligonucleotides and Analogs (1991), IRL Press, New York; Shaw et al. (1993), Methods Mol. Biol.20:225-243; Nielsen et al. (1991), Science 254:1497-1500; and Shaw et al. (2000) Methods Enzymol. 313:226-257.

[0255] Oligonucleotides, including modified oligonucleotides (e.g., oligonucleotides comprising fluorophores and quenchers, unnatural nucleotides, 2'-O-methyl nucleotides, and/or phosphorothioate, methylphosphonate, or boranophosphate linkages) can also be ordered from a variety of commercial sources known to persons of skill. There are many commercial providers of oligo synthesis services, and thus, this is a broadly accessible

technology. Any nucleic acid can be custom ordered from any of a variety of commercial sources, such as The Midland Certified Reagent Company (www.mcrc.com), The Great American Gene Company (www.genco.com), ExpressGen Inc. (www.expressgen.com), QIAGEN (http://oligos.qiagen.com), Dharmacon (www.dharmacon.com), and many others.

[0256] A variety of nuclease-resistant nucleic acids can optionally be created, e.g., comprising modified nucleotides and/or modified internucleotide linkages such as those currently used in the synthesis of antisense oligonucleotides. For example, a nuclease resistant oligonucleotide can comprise one or more 2'-O-methyl nucleotides. For example, an oligonucleotide comprising standard deoxyribonucleotides can also comprise one or more 2'-O-methyl nucleotides (e.g., at its 5' end), or an oligonucleotide can consist entirely of 2'-O-methyl nucleotides. As another example, a nuclease resistant oligonucleotide can comprise one or more phosphorothioate linkages (oligonucleotides comprising such linkages are sometimes called "S-oligos"). An oligonucleotide can comprise, e.g., only phosphorothioate linkages or a mixture of phosphodiester and phosphorothioate linkages. In other embodiments, the oligonucleotide comprises one or more methylphosphonate linkages, one or more boranophosphate linkages, or the like. Combinations of typical nuclease resistance modification strategies can also be employed; for example, a nuclease resistant oligonucleotide can comprise both 2'-O-methyl nucleotides and phosphorothioate linkages.

[0257] As noted, a nucleic acid can be produced by chemical synthesis or can be custom ordered. In addition, nucleic acids can be produced by enzymatic synthesis (in vitro or in vivo). For example, interfering RNAs can be produced by in vitro transcription using techniques well known in the art. Kits for in vitro transcription are commercially available; for example, the Silencer™ siRNA construction kit from Ambion, Inc. (www.ambion.com).

Polypeptide Production

[0258] Polypeptides (e.g., polypeptide cellular delivery modules, e.g., protein transduction domains) can optionally be produced by expression in a host cell transformed with a vector comprising a nucleic acid encoding the desired polypeptide(s). Expressed polypeptides can be recovered and purified from recombinant cell cultures by any of a number of methods well known in the art, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography (e.g.,

using any of the tagging systems noted herein), hydroxylapatite chromatography, and lectin chromatography, for example. Protein refolding steps can be used, as desired, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed in the final purification steps. See, e.g., the references noted above and Deutscher, Methods in Enzymology Vol. 182: Guide to Protein Purification, Academic Press, Inc. N.Y. (1990); Sandana (1997) Bioseparation of Proteins, Academic Press, Inc.; Bollag et al. (1996) Protein Methods, 2nd Edition Wiley-Liss, NY; Walker (1996) The Protein Protocols Handbook Humana Press, NJ; Harris and Angal (1990) Protein Purification Applications: A Practical Approach IRL Press at Oxford, Oxford, U.K.; Scopes (1993) Protein Purification: Principles and Practice 3rd Edition Springer Verlag, NY; Janson and Ryden (1998) Protein Purification: Principles, High Resolution Methods and Applications, Second Edition Wiley-VCH, NY; and Walker (1998) Protein Protocols on CD-ROM Humana Press, NJ.

[0259] Alternatively, cell-free transcription/translation systems can be employed to produce polypeptides encoded by nucleic acids. A number of suitable in vitro transcription and translation systems are commercially available. A general guide to in vitro transcription and translation protocols is found in Tymms (1995) In vitro Transcription and Translation Protocols: Methods in Molecular Biology Volume 37, Garland Publishing, NY.

[0260] In addition, polypeptides (including, e.g., polypeptides comprising fluorophores and quenchers and/or unnatural amino acids) can be produced manually or by using an automated system, by direct peptide synthesis using solid-phase techniques (see, e.g., Stewart et al. (1969) Solid-Phase Peptide Synthesis, WH Freeman Co, San Francisco; Merrifield J (1963) J. Am. Chem. Soc. 85:2149-2154). Exemplary automated systems include the Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer, Foster City, CA). In addition, there are many commercial providers of peptide synthesis services. If desired, subsequences can be chemically synthesized separately, and combined using chemical methods to provide full-length polypeptides.

EXAMPLES

[0261] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of

this application and scope of the appended claims. Accordingly, the following examples are offered to illustrate, but not to limit, the claimed invention.

PA Sensors: Constructs and Methods for Measuring RNA Transcripts in Living Cells

[0262] In one aspect, the present invention provides sensors for detecting and measuring mRNA in living cells (also known as PAC probes for mRNA) and methods of controlling activation of such mRNA sensors in living cells. In one class of embodiments, the sensor is attached to one or more photo-labile groups that protect the sensor from extra-cellular and intra-cellular degradation and, at the same time, inactivate the sensor. Upon exposure to light of a specific wavelength, the photolabile groups detach from the sensor and the sensor becomes active. The mRNA sensors include one or more labels (e.g., a combination of acceptor and donor fluorophores that interact via FRET or ET or a fluorophore/quencher combination) on RNAs that can initiate RNAi (e.g., siRNA, shRNA; Figures 12-14, in which A and B represent either a fluorescent label and a quencher (or vice versa) or a donor and acceptor (or vice versa)). The signal from the sensor is used to detect and measure mRNA in living cells. Splice variants of mRNAs, for example, can also be analyzed using interfering RNA approaches.

[0263] Traditional or novel delivery methods can be used to introduce a sufficient quantity of mRNA sensors into cells. A high throughput uncaging device, such as those described in USSN 60/427,664 filed November 18, 2002, 60/436,855 filed December 26, 2002, 60/439,917 filed January 13, 2003, 60/451,177 filed February 27, and 60/456,870 filed March 21, 2003, can be used to activate photoactivatable sensors, e.g., in cells grown in a microtiter plate. This invention also features methods of detecting and measuring mRNA with such sensors in living cells.

[0264] The ability to monitor immediate changes in mRNA levels in living cells facilitates the development of a broad range of cell-based assays for basic research, pharmaceutical industries, clinical and agricultural diagnostics. For example, a specific GPCR or kinase cell-based assay can be developed for screening lead compounds using one or more PAC probes for monitoring mRNAs downstream of the GPCR or kinase. Actual transcript or surrogate transcript (marker, mRNA of a gene further downstream in a pathway) response to modulation of specific pathways by the compounds can be monitored in living cells.

[0265] An example PAC probe for an mRNA comprises a labeled interfering RNA (e.g., an siRNA or a shRNA; see, e.g., Watanabe (Jan.13 2003) Scientist 17(1):36; D Engelke (2002) Nature Biotech 29: 505; Trends in Biotech 20:49 (2002)); one or more caging groups, e.g., photolabile caging groups (see, e.g., FR Haselton JBC 274:20895 and H Okamoto (2001) Nature Genetics 28:317); and optionally a cellular and/or subcellular delivery module, e.g., a peptide delivery module such as TAT or Antp (see, e.g., Lane (2001) Bioconjugate Chemistry 12:825).

[0266] Modified nucleotides can optionally be incorporated into interfering RNAs to reduce degradation in cells. For example, a phosphate backbone analog (e.g., phosphorothioate and/or a modified nucleotide (for example, a 2'-O-methyl nucleotide, e.g., 2'-O-methylinosine) can be used to protect the RNA from nuclease digestion. Caging groups can also protect against nuclease digestion.

[0267] Figure 12 describes a small interfering RNA (siRNA, also known as short interfering RNA) structure used for detecting mRNA in living cells. The siRNA can be, for example, a 21-25 mer double-stranded RNA; other lengths and/or optional overhangs (e.g., two nucleotide 3' overhangs) can also be used. A reporter combination (e.g., a fluorophore/quencher pair or acceptor/donor FRET pair) is linked at the 5' and 3' ends of one strand or at the ends of opposite strands. The reporter molecules can also be within the siRNA, either on the same strand or on opposite strands of the double stranded siRNA. The reporters can be, e.g., a combination of FRET dyes such as coumarin and FITC or a combination such as europium and APC that permits application of time-resolved fluorescence (TRF) techniques.

[0268] An interfering RNA can be caged, e.g., with photo-labile groups, at the phosphates, riboses and/or bases to protect it and to inactivate its function. It can optionally be linked to a delivery module, e.g., a peptide delivery module (for example, 8-D-Arg, Antp, Pep-1, or the like), e.g., with a disulfide linker as illustrated in Figure 14. Other established delivery approaches can also be used, e.g., lipofection.

[0269] Figure 13 shows another type of interfering RNA for measuring mRNA, e.g., a short hairpin RNA (shRNA, also called small hairpin RNA; e.g., Nature Genetics 33:396). For example, a shRNA can have about 60-70 nt that form a hairpin, e.g., with a 25-30 mer double-stranded region and an 8 mer single-stranded loop. A reporter combination (e.g., a

donor and an acceptor fluorophore that interact via FRET, or a fluorophore/dark quencher) can be attached for signaling the presence of a specific RNA transcript. As in the previous example, the shRNA can be caged, e.g., with photolabile caging groups.

[0270] Figure 15 shows the detection of mRNA using an interfering RNA PAC probe. The siRNA is incorporated into the RISC complex, and the antisense strand guides cleavage of the target mRNA (promoting its degradation). Strand separation of the interfering RNA probe leads to the separation of the reporter molecules on the RNA, resulting in a detectable signal or change in signal (as indicated by the starburst symbol). Multiple mRNA transcripts can be analyzed using interfering RNAs with different reporter molecules (e.g., fluorophores that emit at different wavelengths).

[0271] Figure 16 shows the detection of a single target using multiple (e.g., two or more) interfering RNA sensors. The different interfering RNAs typically emit distinguishable signals before and/or after initiation of RNAi. Detection specificity is improved using this design, because an actual signal or signal change (indicating degradation of the specific target mRNA) is recorded only when signals from both interfering RNAs are observed at about the same time.

Applications

[0272] Figure 17 shows an example workflow for mRNA measurement using the sensors of this invention, where the effect of a compound (drug, agonist, antagonist, etc. affecting or potentially affecting an upstream signaling molecule) on mRNA level is monitored. There are minimal fluidic handling steps and reagents required. A photolabile PAC probe can be uncaged by exposing to a light source (e.g., in an uncaging device such as those described in USSN 60/427,664 filed November 18, 2002, 60/436,855 filed December 26, 2002, 60/439,917 filed January 13, 2003, 60/451,177 filed February 27, and 60/456,870 filed March 21, 2003).

[0273] A PAC probe for mRNA can be used to measure amount of mRNA transcript and location of mRNA processing in living cells. When performing quantitative analysis, an interfering RNA sensor for a house-keeping RNA can optionally be used to normalize for variable target(s). Deviation between different cells can be corrected if one or more dual-labeled FRET interfering RNAs, for example, are used instead of a dark quencher/fluorophore probe format. With a dual-labeled FRET probe (i.e., a probe with a

donor fluorophore and an acceptor fluorophore, where the donor and acceptor are capable of exhibiting FRET), at least two different signals can be obtained, i.e., the FRET signal (emission by the acceptor following stimulation of the donor) and the acceptor signal (emission by the acceptor following stimulation of the acceptor) using different excitation wavelengths, e.g., produced by different lasers, to stimulate the donor and acceptor. The ratio of these two signals can be taken, e.g., to normalize for transfection efficiency of the probe.

[0274] As noted, interfering RNA PAC probes can be used to analyze splice variants (including, e.g., in living cells). Examples of genes with a variety of splice variants are beta-actin and cyclic nucleotide phosphodiesterases (Current Opinion in Cell Biology (2000) 12:174-179), among many others. To analyze alternatively spliced mRNAs, for example, a siRNA probe can be designed to recognize the splice junction. One or more such siRNAs can be used to detect various isoforms. For example, Figure 18 illustrates how multiple siRNAs can be used to determine splice variants. A nuclear RNA containing three exons and two introns is transcribed from chromosomal DNA. The nuclear RNA is spliced to form the mRNA, which in this example includes all three exons and no introns. A siRNA is designed to be at the splice junction. The isoform with the correct splice variant is digested. Similarly, siRNA can be made to bind to the exon regions and not between the splice junctions, or a siRNA can be designed against an intron. Splice variants containing the intron are digested and result in a signal from the siRNA probe.

Cell based assay using labeled interfering RNA as in vivo mRNA sensor

[0275] The following sets forth a series of experiments that demonstrate design and use of interfering RNA sensors to detect GAPDH mRNA. GAPDH is constitutively expressed.

[0276] Three different interfering RNAs were designed against GAPDH (Figure 19; SEQ ID NO:1): RNAi 1, corresponding to nt 690-708 (each strand has 19 GAPDH bases plus a TT 3' overhang), RNAi 2, corresponding to nt 915-936 (each strand has 21 GAPDH bases, forming a 19 bp double-stranded region and two nucleotide overhangs), and RNAi 3, corresponding to nt 601-621 (each strand has 21 GAPDH bases, forming a 19 bp double-stranded region and two nucleotide overhangs). Each RNA was labeled with 6-FAM on the 3' end of the antisense strand, and a Dabcyl quencher was attached to the 3' end of the sense strand. The FAM label and Dabcyl quencher were incorporated during oligonucleotide

synthesis. Figure 20 illustrates one of the three GAPDH RNAi sensors. When the sense and antisense strands are annealed, the FAM label is quenched (Panel A); when the strands are denatured, the label is not quenched and fluoresces (Panel B). Panel C shows fluorescent emission spectra for the antisense strand (curve 1), the sense strand (curve 2), and the annealed strands (curve 3), illustrating that the FAM label is quenched in the annealed sensor.

[0277] To verify that the labeled RNAs were able to attenuate expression of GAPDH, labeled RNAi 1-3 were lipofected into HeLa cells (1000 cells) at a concentration of 0.5 μg/μl for 4 hours. Cells were maintained at two temperatures (37°C and 45°C) and lysed at different time points after lipofection (4 h, 10 h, 20 h, 34 h, and 44 h). GAPDH mRNA was measured using a branched DNA (bDNA) assay (see, e.g., Journal of Clinical Virology (2002) 25:205-216; QuantiGene bDNA assay kits are commercially available from Genospectra, Inc., www.genospectra.com).

[0278] Figure 21 shows the GAPDH mRNA level as measured by the bDNA assay at the indicated time points after lipofection of labeled RNAi 1 (Panel A), as compared to a negative control (Panel B, no lipofection reagent). Figure 22 compares the percentage knockout of GAPDH expression, as measured by the bDNA assay, for labeled RNAi 1-3. RNAi 1 was the most potent silencer of the three interfering RNAs tested, knocking out GAPDH expression in HeLa cells by as much as 90%.

[0279] To test the labeled RNAi's as in vivo mRNA sensors, the three GAPDH RNAis (labeled with 6-FAM and Dabcyl) were lipofected into HeLa cells (1000 cells) at a concentration of 0.5 μg/μl for 4 h at 37°C. The cells were incubated with fresh medium at 37°C. Cells were fixed 4 h and 20 h after lipofection and scanned on a Packard scanner for FITC signal, and bDNA assays were performed at the same time points. Figure 23 shows the results of the bDNA assays (RLU, luminescence) compared to the FITC signals (FLU) for cells lipofected with the RNAi 1 (Panel A), RNAi 2 (Panel B), and RNAi 3 (Panel C) sensors. We observed opposing trends over time between the signals for the labeled RNAi sensors (increased FITC signal, reflecting degradation of GAPDH mRNA) and the bDNA data (reduced GAPDH mRNA level in the presence of interfering RNA).

[0280] Figure 24 shows the ratio of the bDNA assay measurement of GAPDH mRNA levels at 20 h/4 h and the ratio of the FITC signal from labeled RNAi's 1-3 at 20 h/4

h, and demonstrates that RNAi 1 is the most effective in silencing GAPDH and the most prominent in generating FRET signal.

[0281] Labeled RNAi 1 was further tested as an in vivo mRNA sensor. 2000 HeLa cells were plated in each well in eight well chambers with complete DMEM medium overnight at 37°C. Medium was changed to OptiMEM, and the cells were lipofected with GAPDH RNAi 1 (2 μg, 4 μg) for 4 h in reduced serum medium at 37°C. At three different time points (0 h, 4 h, and 10 h after lipofection), duplicate slides were plated. One slide was used for a bDNA assay, the other for scanning the FAM signal. For the bDNA assay, cells were lysed with bDNA lysis buffer at 0 h, 4 h, and 10 h time points, and lysate from approximately 300 cells was assayed for GAPDH mRNA using the bDNA assay. (Note that "0 h" is the time point after the lipofection process, which takes about 4 hours.) At each time point, the duplicate slide was fixed and scanned on a Packard scanner in the FAM channel at 90% power and 70% PMT gain.

[0282] Fluorescent signal from the RNAi 1 sensor increased over time (from 0 to 4 h and from 4 to 10 h) at both amounts of sensor tested (data not shown). Fluorescent signal from the sensor also increased with increasing amount of sensor; 4 μg of RNAi 1 produced a more intense signal than 2 μg at each time point. At both amounts of RNAi 1 tested (2 μg and 4 μg), the level of GAPDH mRNA as measured by the bDNA assay typically decreased over time.

[0283] An additional test of labeled RNAi 1 as an in vivo mRNA sensor was performed. 2000 HeLa cells were plated in each well in eight well glass slides with complete DMEM medium overnight at 37°C. Medium was changed to OptiMEM, and the cells were lipofected with 4 μg of GAPDH RNAi 1 for 4 h in reduced serum medium at 37°C. At three different time points (0 h, 4 h, and 10 h after lipofection), duplicate slides were plated. One slide was used for a bDNA assay, the other for scanning the FAM signal. For the bDNA assay, cells were lysed with bDNA lysis buffer at 0 h, 4 h, and 10 h time points, and lysate from approximately 350 cells was assayed for GAPDH mRNA using the bDNA assay. (Note that "0 h" is the time point after the lipofection process, which takes about 4 hours.) At each time point, the duplicate slide was fixed and scanned on a Packard microarray scanner in the FAM channel at 90% power and 60% PMT gain; for each well, fluorescent signal from the entire well was analyzed. The experiment was performed in

duplicate on three independent days, and the resulting data were averaged to obtain the results plotted in Figure 25.

[0284] Figure 25 Panel A shows the results of the bDNA assay (diamonds, RLU, representing the GAPDH mRNA level in the cells at the indicated time points) and the fluorescent signal for the labeled RNAi 1 mRNA PAC probe (circles, RFU), at each time point. Figure 25 Panel B plots the fluorescent signals from the labeled RNAi 1 sensor against the results of the bDNA assay. We note an inverse linear relationship between the signal from the interfering RNA sensor and the amount of GAPDH mRNA remaining in the cells.

[0285] It is worth noting that in the examples above, the level of fluorescent signal from the siRNA sensor is correlated to the cumulative destruction of GAPDH mRNA in the cells. As more GAPDH mRNA gets degraded, the signal from the sensor increases. (Clearly, in these examples, the increase in sensor signal level from 0 h to 4 h to 10 h (e.g., for the RNAi 1 sensor in Figure 25) does not mean that the GAPDH mRNA level is increasing.) Therefore, for transcripts already abundant in cells (e.g., constitutively expressed genes, such as GAPDH), an siRNA sensor can provide an indication of the knock-down efficiency of the siRNA. The methods can similarly be applied to determine the knock-down efficiency of an siRNA against an inducible target mRNA.

[0286] In summary, we conclude from the above experiments that the magnitude of the FRET signal for the FAM label on the RNA sensor correlates to the level of GAPDH expression knockdown as measured by the bDNA assay and inversely correlates with the level of GAPDH remaining in the cell, confirming that labeled GAPDH interfering RNA functions as an inhibitor sensor.

Discussion

[0287] It will be evident to one of skill that the methods of detecting target mRNA in a cell using a labeled interfering RNA sensor described herein have a number of applications, and that the signal output detected from the sensor can provide different types of information under different circumstances. The signal output is typically proportional to the amount of target mRNA degraded; depending on the circumstances, the signal output can be, e.g., proportional to the amount of target mRNA initially present or induced in the

cell and/or inversely proportional to the amount of mRNA remaining in the cell, as illustrated in the following examples.

[0288] For example, the methods can be used to determine how effective any given siRNA is at knocking down (or knocking out) expression of its target mRNA, e.g., in real time in living cells. The siRNA can be labeled to produce an siRNA sensor, which can be used in the methods described herein. For example, in the experiments described above, the signal output from RNAi 1 is stronger than that from RNAi 2, indicating that RNAi 1 leads to the degradation of more GAPDH mRNA then RNAi 2, and thus indicating that RNAi 1 is better at knocking down GAPDH expression then is RNAi 2 (see, e.g., Figs. 22-24).

[0289] As another example, the methods can be used for real-time, dynamic monitoring of target mRNA levels. The experiment summarized in Figure 25, for example, illustrates an inverse linear relationship between target mRNA levels and signal output from the sensor. In this example, a stronger fluorescent signal from the sensor indicates more of the constitutively expressed GAPDH transcript has been degraded and thus that less of the transcript is currently present in the cell.

[0290] The methods can be used to monitor both constitutively expressed and/or inducible target mRNA levels. Thus, in yet another example, the methods can be used to detect expression of an inducible gene, e.g., in real time in living cells. For example, an siRNA sensor for an inducible target gene (e.g., IL-8) can be introduced into cells, expression of the target gene can be induced, and the level of signal from the siRNA sensor (e.g., the slope, intercept(s), and/or maximum value(s) from a plot of signal strength versus time) can be used as an indication of the onset of target gene expression and/or the degree of induction of the target gene. In this example, the level of fluorescent signal from the siRNA sensor is correlated to the degree of induction. As more target gene transcript becomes available for RNA interference, the sensor signal increases. Again, the level of sensor signal reflects the amount of transcript being destroyed and not the final level of the inducible transcript. Since the amount of transcript destroyed is proportional to the degree of induction of the inducible gene, the level of sensor signal is proportional to the degree of induction: a stronger signal indicates stronger induction (more transcripts destroyed).

[0291] As yet another example, a caged siRNA sensor can be used in the methods to detect the mRNA level of a target gene, e.g., in real time in living cells. The caged siRNA

sensor is put into the cells and is then uncaged (e.g., at a preselected time). The level of signal from the sensor can be used as a measurement of the transcript level immediately prior to uncaging. Preferably, in these example embodiments, the concentration of the caged siRNA sensor is higher than the concentration of the target mRNA. The slope, intercept(s), and/or maximum value(s) from a plot of signal strength versus time after uncaging, for example, can be used to reflect the target mRNA level at the time of uncaging. Again in this example, a stronger signal from the sensor indicates degradation of more target mRNA and thus a higher concentration of the target mRNA in the cell at the time of uncaging.

In vivo photoactivation of photolabile caged siRNA

[0292] The following sets forth a series of experiments that demonstrate use of a photolabile caged siRNA to control initiation of RNAi of the GAPDH mRNA. The 5' phosphate of the antisense strand of RNAi 1 was caged (Figure 28). The caged antisense oligo (5' PhotoCageAGUAGAGGCAGGGAUGAUGdTdT 3', SEQ ID NO:2) was synthesized by Trilink Biotechnologies, Inc. (www.trilinkbiotech.com), as follows. The commercially available caged phosphoramidite [1-N-(4,4'-Dimethoxytrityl)-5-(6-biotinamidocaproamidomethyl)-1-(2-nitrophenyl)-ethyl]-2-cyanoethyl-(N,N-diisopropyl)-phosphoramidite (PC Biotin Phosphoramadite, from Glen Research Corp., www.glenres.com) was coupled to the 5' terminus of a 21-mer oligoribonucleotide using standard phosphoramidite chemistry. Following the coupling step, oxidation, and cleavage from the resin, the caged oligoribonucleotide was purified using RNase-free HPLC purification and verified using gel electrophoresis analysis and mass spectrometry. An oligoribonucleotide corresponding to the sense strand was also synthesized (5' CAUCAUCCCUGCCUCUACUdTdT 3', SEQ ID NO:3), and equimolar amounts of the sense and caged antisense strands were annealed to form the caged RNAi 1. An RNAi 1 siRNA which did not contain the caging group was also synthesized.

[0293] HeLa cells were lipofected with 100 nM RNAi 1, caged RNAi 1, or caged RNAi 1 that had been uncaged in vitro, using Lipofectamine™ 2000 (Invitrogen, www.invitrogen.com) according to the manufacturer's instructions. In brief, 5000 HeLa cells were plated evenly into each well of 96 well Corning Costar black clear bottom plates in 200 μL of Dulbecco modified Eagle medium (DMEM). The cells were incubated at 37° C for 16-24 h, and then visually examined to ensure that each well was 70-90% confluent

and that the culture was evenly distributed in each well. For each well, 0.25 μg of the appropriate siRNA was diluted in 25 μL OptiMEM and incubated at room temperature for 4 min; 0.5 μL of Lipofectamine™ 2000 was also diluted in 25 μL OptiMEM and incubated at room temperature for 4 min. The siRNA and the lipofection reagent were then combined, mixed gently, and incubated at room temperature for 20 min, the volume was adjusted to 175 μL with OptiMEM, medium was aspirated from the well containing the HeLa cells, and the siRNA-lipofection reagent complex was added to the cells. Plates were then incubated for 4 h at 37° C with gentle shaking, then the medium was replaced with 200 μL fresh complete DMEM.

[0294] To cleave the caging group from the caged siRNA, following the 4 h lipofection with caged RNAi 1, cells were exposed from the bottom of the well to 1 J/cm^2 365 nm UV light. Uncaging light was produced by a BlueWave™ UV Spot Light System fitted with a Lightguide mount assembly, Cool Blue™ filter, and Lightguide rod lens assembly (Dymax Corp., www.dymax.com, part numbers 38600, 38670, and 38699). Cells were incubated at 37° C and lysed at different time points after uncaging: 0 h (immediately after uncaging), 6 h and 10 h after uncaging.

[0295] As controls, cells lipofected with unmodified RNAi 1, cells lipofected with caged RNAi 1 but not exposed to uncaging light, and cells lipofected with caged RNAi 1 that had previously been uncaged in vitro by exposure to 12 J/cm^2 of UV light were also maintained at 37° C and lysed at 0 h (immediately following lipofection, corresponding to the 0 h time point for the in vivo uncaged caged siRNA above), 6 h, and 10 h.

[0296] GAPDH mRNA was measured with a branched DNA assay using a Quantigene Explore bDNA assay kit (Genospectra, Inc.) according to the instructions supplied with the kit. To normalize for cell number, GAPDH expression was normalized to cyclophilin expression (also measured with a bDNA assay).

[0297] Figure 29 Panel A shows GAPDH expression normalized to cyclophilin expression in untransfected cells and cells transfected with: RNAi 1 (unmodified), in vitro uncaged caged RNAi 1, caged RNAi 1 (transected cells were not exposed to light), and in vivo uncaged caged RNAi 1, as measured by the bDNA assay at the indicated time points after uncaging (or just lipofection). Figure 29 Panel B shows the relative GAPDH mRNA level as measured by the bDNA assay at the indicated time points after uncaging (or just

lipofection) of: RNAi 1 (unmodified), in vitro uncaged caged RNAi 1, caged RNAi 1 (transected cells were not exposed to light), and in vivo uncaged caged RNAi 1. Expression is normalized to that of cells transfected with the unmodified RNAi 1. Comparing relative GAPDH expression in cells transfected with unmodified RNAi 1 and caged RNAi 1 indicates that the caging group inhibits initiation of RNAi by the caged siRNA; levels of GAPDH mRNA are higher for cells transfected with the caged siRNA but not exposed to light than for cells transfected with unmodified RNAi 1 at all three time points. Removal of the caging group restores the ability of the siRNA to participate in RNAi, since relative GAPDH levels in cells transfected with caged RNAi 1 and then exposed to UV light are close to the levels in cells transfected with RNAi 1 at 6 and 10 h after uncaging.

[0298] While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques and apparatus described above can be used in various combinations.

[0299] All publications, patents, patent applications, and/or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document were individually indicated to be incorporated by reference for all purposes.